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OVOMUCIN, A SUBSTRATE FOR THE ENZYME OF INFLUENZA VIRUS. I. OVOMUCIN AS AN INHIBITOR OF HAEMAGGLUTINATION BY HEATED LEE VIRUS.

A. GOTTSCHALK AND PATRICIA E. LIND.

From the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

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IT was previously shown by Hirst (1942) (1) that influenza viruses are adsorbed to chicken red blood cells and elute spontaneously after a period of time, the process of elution having a temperature coefficient of about 3 over the range 27–37° C ; (2) that the virus after its spontaneous elution from the cell surface is functionally intact, whereas the red cell is irreversibly changed so as to be no longer available for virus adsorption. In interpreting his exacting observations Hirst compared the interaction between the virus agglutinin and the receptor substance at the surface of the red cells with an enzyme-substrate interaction, the enzyme possessed by the virus changing the properties of the cell receptor substance.

In a series of investigations Burnet and co-workers (Burnet, McCrea and Stone, 1946 ; Stone, 1947 ; Anderson, 1948 ; Burnet, 1948*a* ; Burnet, 1948*b*) have advanced much evidence to substantiate Hirst's suggestion of the presence at the virus surface of a true catalyst. The method used by Burnet to test for virus enzyme activity is based on the findings (1) that influenza virus type B, when heated at 55° C. for 30 minutes, fully retains its haemagglutinating properties, but loses its ability to elute spontaneously (Briody, 1948) ; (2) that certain substances are able to inhibit haemagglutination by heated virus, when mixed together with the virus prior to the addition of the red blood cells. The first observation relevant to (2) was made by Francis (1947), who showed the power of normal human serum to inhibit haemagglutination by heated influenza B virus. McCrea (1948) proved the inhibitor present in serum to be a component of the heat-stable mucoprotein fraction of human serum and prepared (McCrea, 1949) from ovarian cyst fluid a purified mucopolysaccharide highly active as "virus haemagglutinin inhibitor," i.e. a substance able to combine with and to fix heated influenza B virus, thereby inhibiting the agglutination of added red blood cells. Burnet (1948*a*) demonstrated the inhibitory power of purified blood group O substance, a mucopolysaccharide prepared from human ovarian cysts

by Morgan (King and Morgan, 1944 ; Morgan, 1947 ; Aminoff and Morgan, 1948), and de Burgh, Yu, Howe and Bovarnick (1948) provided evidence for the polysaccharide nature of a very efficient inhibitor which can be extracted from human red cells and may be identical with the receptor substance of erythrocytes (Hirst, 1948).

The important fact disclosed by Burnet and co-workers (Anderson, 1948 ; Burnet, 1948*a*, 1948*b*) is the loss by previous treatment with active (unheated) influenza virus of the ability of the various mucoproteins and mucopolysaccharides to act as virus haemagglutinin inhibitor. Whatever may be the forces of attraction between the surface of the heat-inactivated virus and the mucoid molecule, the lack of association after treatment with active virus scarcely allows of another interpretation than of a structural change in the substrate. This finding, taken together with the further observation that a soluble enzyme of *Vibrio cholerae*, referred to as receptor destroying enzyme or RDE (Burnet and Stone, 1947), closely imitates active influenza virus in its ability to reduce the power of mucoids to inhibit haemagglutination by heated virus (Anderson, 1948 ; Burnet, 1948*a*), strongly suggests the enzymatic nature of the process.

At this stage it seemed desirable to investigate the chemical change involved in the interaction between active influenza virus and suitable mucoproteins. It is the aim of the experimental work to be described in a series of papers to present information regarding the composition of the product of this interaction, thus affording conclusive evidence for the enzymatic character of the virus activity under discussion and obtaining some insight into the chemistry of the underlying reaction. For such an investigation it was essential to provide a virus inhibitor easily available and readily acted upon by the virus concerned. In the search for such a substance use was made of the observation by Burnet (unpublished) that hen egg-white is an efficient virus haemagglutinin inhibitor, a property rapidly lost by pre-treatment with active virus or with RDE. Lanni and Beard (1948) have also reported a similar inhibitory effect of egg-white on haemagglutination by heated swine influenza virus. In this paper it will be shown that of the various egg-white proteins the ovomucin fraction is mainly responsible for these phenomena. Since the preparation of this fraction presents no difficulty, ovomucin fulfils the specified requirements.

MATERIALS.

Preparation of ovomucin.

Ovomucin was prepared following mainly the procedure described by Young (1937). 600 ml. egg-white, obtained from about 20 fresh white Leghorn eggs and freed of the chalazae, were chilled and poured into 1600 ml. distilled water at 0°C. The precipitate was centrifuged off and washed three times with cold 0·5 per cent NaCl solution in the centrifuge. The washed precipitate was dispersed in the minimal volume of 10 per cent NaCl solution and reprecipitated by adding the dispersion to about twenty volumes of distilled water at 0° C. After centrifugation the snow-white precipitate was washed with water until washings were chlorine free and practically devoid of protein. The final product was dried over conc. H_2SO_4 *in vacuo*. This preparation, of a white, silky appearance was analyzed for moisture, ash, nitrogen, hexosamine and reducing substances with the following result :

TABLE I.—*Analysis of the Ovomucin Fraction of Egg-white.*

	Moisture. mg.	Ash. mg.	Nitrogen. mg.	Reducing substance (as glucose). mg.	Hexosamine. mg.
100 mg. ovomucin contain	6·3	1·64	13·20	15·4 (15·1*)	11·8

* Hydrolysed with 2N HCl at 100° C. for 5 hours.

The figures in Table I are in fair agreement with the data given by Young (1937). Ovomucin, once dried, even if drying is gently effected from the frozen state, is scarcely soluble in water or in NaCl solutions of various concentration. It was found that the capacity of ovomucin to inhibit haemagglutination by heated virus and the loss of this capacity by pre-treatment with active virus was changed neither quantitatively nor qualitatively, if the precipitates were washed only once. Therefore, for experiments concerned mainly with the kinetics of the virus-substrate interaction the second precipitate, after one washing, was redispersed in an appropriate volume of NaCl solution to give a final ovomucin concentration of 0·5 to 1·0 per cent and a final salt concentration of 5 per cent. This dispersion, showing a considerable viscosity and a faint opalescence, was dialysed in a cellophane tube for 48 hours at 4° C. against two changes of 1·8 and 1·0 per cent NaCl solution respectively.

It must be pointed out, however, that ovomucin, prepared from egg-white by precipitation with water, represents a protein fraction rather than an individual protein. As was shown in this Institute by Mr. E. L. French, all ovomucin preparations contain lysozyme in various concentration, up to 15 per cent of the total ovomucin fraction. The lysozyme content of this fraction may be considerably decreased by shifting the pH of the final dispersion (in 5 per cent NaCl) to 9·7 with 1 N KOH, chilling and seeding the liquid with crystals of isoelectric lysozyme. Under these conditions lysozyme crystallizes out from the dispersion within 2 or 3 days at 4° C. and is removed together with some gelatinous matter by centrifugation. By this method, described for egg-white by Alderton and Fevold (1946,) the lysozyme content of the ovomucin fraction can be reduced to about 4 per cent of the total. The dispersion, after readjustment of the pH to 7·0, is then dialysed, as described above. This procedure yields a rather uniform dispersion of the ovomucin fraction, which may be diluted with 0·85 per cent NaCl solution, if so required.

It may be mentioned that separation or concentration of the virus haemagglutinin inhibitor component of the ovomucin fraction by chloroform extraction (Sevag's (1934) method as modified by Bay, Henry and Stacey (1946)) is not feasible. If an ovomucin dispersion (inhibitory titre 6400) was shaken for 30 minutes with 1/5 of its volume of chloroform and 1/50 of its volume of *n*-butanol and then centrifuged for 5 minutes, only 5 per cent of the inhibitor was recovered in the upper layer (inhibitory titre 300). Submitting the upper layer to the same treatment further reduced the inhibitory power (inhibitory titre <50).

Preparation of ovomucoid, egg albumin and lysozyme.

Ovomucoid was prepared according to Mörner (1894), crystalline egg albumin according to Cole (1933) and crystalline lysozyme (isoelectric) from egg-white according to Alderton and Fevold (1946).

Purification and concentration of influenza virus.

Two strains of influenza virus, Melbourne (type A) and Lee (type B) were used. In each case purification and concentration were carried out in the following manner: 75 ml. of a centrifuged pool of allantoic fluids harvested from 13-day-old chick embryos, inoculated with the respective virus 2 days previously, were mixed with 75 ml. of 0·1 M phosphate buffer (pH = 8·2). 0·1 M calcium chloride solution was then added drop by drop with constant stirring in two portions of 6·8 ml. and 4·5 ml. respectively and the precipitate resulting from each addition spun down in a refrigerated centrifuge. After washing with 0·1 M phosphate buffer (pH = 8·2) the precipitate was quickly dissolved in 10 ml. of half-saturated ammonium citrate solution, previously adjusted to pH 6·3 with citric acid, and the mixture dialysed for 60 minutes against running tap water and for 48 hours at 4° C. against two changes of 4 l. 0·8 per cent NaCl solution. The whole procedure was performed under sterile conditions, and only preparations free from bacterial contamination were used in the experiments. The technique is a modification of Salk's (1941) method, first applied in this Institute by Dr. McCrea.

Preparation of the receptor destroying enzyme (RDE).

A culture filtrate of a suitable strain of *Vibrio cholerae* was heated at 55° C. for 30 minutes in the presence of excess calcium. From this material a red cell eluate was prepared according to Burnet and Stone (1947).

METHODS.

Nitrogen determinations were made by the micro-Kjeldahl method, using the apparatus of Parnas and Wagner. Period of combustion was 8 hours after the addition of the catalyst (selenium dioxide).

The reducing power was determined by the Hagedorn-Jensen micromethod after hydrolysis of the substance with 1·5 N HCl at 100° C. for 3½ hours in a sealed tube.

The hexosamine content of ovomucin was estimated according to Elson and Morgan (1933). Hydrolysis prior to the determination was effected by 0·5 N HCl at 100° C. for 40 hours, conditions found to give maximum value. The figures, both for the reducing power and the hexosamine content, as tabulated, are not corrected for the unavoidable loss of carbohydrate in the course of acid hydrolysis under the conditions prevailing.

Titration of inhibitory activity of ovomucin.

Titrations were performed essentially according to the technique described by Burnet (1948*a*). Serial twofold dilutions of the inhibitor, with 0·85 per cent NaCl as diluent, were prepared in 0·25 ml. volumes. To each tube was added one drop of Lee infected allantoic fluid, previously heated at 56° C. for 30 minutes and diluted so as to contain five agglutinating doses of virus per drop. After shaking, the mixtures were held for 30 minutes at room temperature, when 0·25

ml. of 1 per cent suspension of "sensitive" fowl red cells (Anderson, 1948) was pipetted into each tube. The cells were then allowed to settle for one hour at room temperature, and the end-point, i.e. 50 per cent haemagglutination, was determined by observation of the pattern of the deposited cells. The reciprocal of the inhibitor dilution showing the end-point was recorded as the inhibitory titre of the preparation. The dilutions were referred to that inhibitor concentration actually used in the experiment, unless otherwise stated.

For the determination of the residual inhibitor in the presence of virus the sample was diluted 1 : 10 with 2 per cent NaCl solution (unless stated otherwise) and heated at 65° C. for 30 minutes to destroy the virus. Controls were treated identically. Titrations were then carried out as above. The inhibitor was only slightly affected by this heat-treatment.

Titration of virus haemagglutinin.

Haemagglutinin titrations of virus were performed according to the method of Burnet, Beveridge, Bull and Clark (1942) with the modification that a suspension of 1 per cent fowl red cells was used.

Titration of RDE activity.

The activity of RDE was determined, as described by Burnet and Stone (1947).

EXPERIMENTAL.

The inhibitory power of ovomucin as compared with that of the other egg-white proteins.

TABLE II.—*The Virus Haemagglutinin Inhibitory Titres of the Various Egg-white Proteins.*

Protein	Inhibitory titre referred to 1 gm. of protein (dry weight)
Ovomucin fraction .	$2{\cdot}4 \times 10^6$ to $4{\cdot}8 \times 10^6$
Ovomucoid . .	1×10^3
Ovalbumin . .	$<2 \times 10^2$
Lysozyme . .	$<2 \times 10^2$

It may be seen from Table II that of the various proteins present in egg-white it is the ovomucin fraction which is endowed with the power to inhibit haemagglutination by heated influenza virus. As will be shown in a following paper, this inhibitory power is a property of only a small portion of the ovomucin fraction. Ovomucin, as usually prepared, is not an individual protein, but rather a protein-mucoprotein mixture. The lysozyme content of the ovomucin fraction can be reduced, as described under "materials." Its complete removal was not attempted, since the residual lysozyme, while representing only a minor impurity, most efficiently protects ovomucin against contamination by air-borne bacteria. It is due to the lysozyme content of ovomucin that larger amounts of this fraction could be prepared without taking sterile precautions and that the incubation of the final product with virus at 38° C. for several hours did not require the addition of an antiseptic.

Reduction of the inhibitory power of ovomucin by active virus.

An approximately 1 per cent dispersion of ovomucin in 1 per cent NaCl solution, the ovomucin concentration calculated on the basis of nitrogen determination, was diluted 1 : 4 with 0·85 per cent NaCl solution, well mixed and the respective virus added to give a final concentration of 40 to 60 agglutinating doses per ml. The mixture and an appropriate control were held in a water-bath at 37° C. Samples were withdrawn at suitable intervals for determination of the residual inhibitory titre.

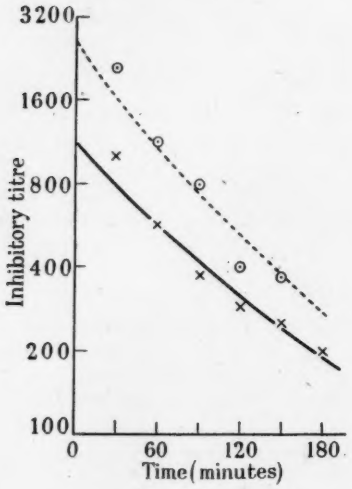

FIG. 1.

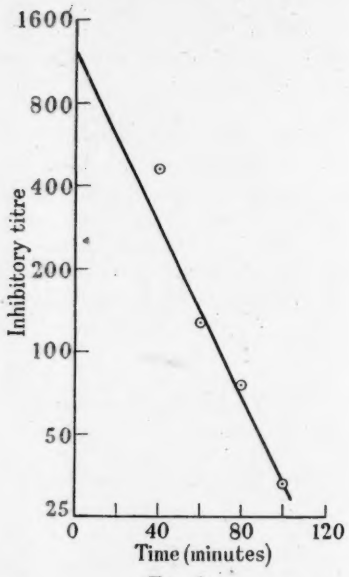

FIG. 2.

FIG. 1.—Action of active influenza virus on the virus inhibitor of ovomucin.
×——× Type A virus (Melbourne).
⊙ - - - - ⊙ Type B virus (Lee).

FIG. 2.—Action of the receptor destroying enzyme (RDE) on the virus inhibitor of ovomucin.

The results of two experiments, one carried out with influenza type A (Melbourne), the other with type B (Lee), are shown in Fig. 1. As may be seen from the slope of the lines, the inhibitory power of ovomucin is rapidly reduced by small quantities of active virus.

Reduction of the inhibitory power of ovomucin by RDE.

A corresponding technique, substituting RDE for virus and adding $CaCl_2$ in M/200 concentration, was used to investigate the effect of RDE on the inhibitory power of ovomucin. The final concentration of RDE in the reaction mixture was 80 units per ml. In order to determine the residual inhibitory titre of ovomucin, RDE was inactivated by preparing the serial dilutions in 0·85 per cent NaCl solution containing 0·005 M sodium hexametaphosphate as calcium deionizing agent. Fig. 2 illustrates the marked decrease in inhibitory titre of ovomucin by the action of RDE.

Reduction of the inhibitory power of ovomucin by trypsin.

The virus haemagglutinin inhibitor of the ovomucin fraction is very sensitive to trypsin action (Fig. 3). The loss by tryptic digestion of the capacity of ovomucin to inhibit haemagglutination by heated Lee virus is in agreement with the finding that the inhibitory power of normal serum is also rapidly destroyed by trypsin treatment (Burnet, McCrea and Anderson, 1947; Hirst, 1948; McCrea, 1948).

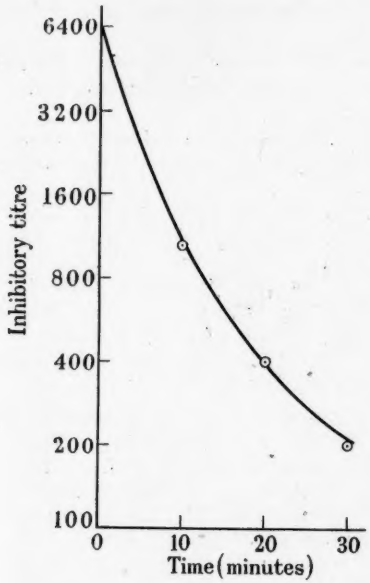

FIG. 3.

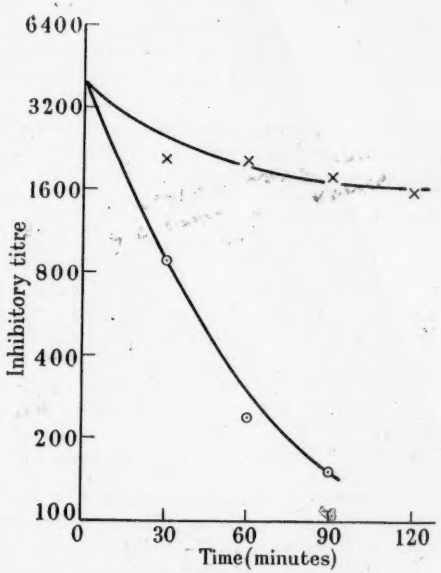

FIG. 4.

FIG. 3.—Action of trypsin on the virus inhibitor of ovomucin.

Conditions: To 1·0 ml. of 1 per cent ovomucin dispersion (in 0·85 per cent NaCl) added 1·0 ml. of 0·1 M phosphage—saline buffer (pH = 7·6), containing 4 mg. crystalline trypsin. Temperature 37° C. Samples for determination of residual inhibitory titre withdrawn at 10 min. intervals.

FIG. 4.—Reduction by active Lee virus of the inhibitory titre of ovomucin with NaCl concentration as the variable.

⊙———⊙ 0·05 ml. active Lee virus added to 1·95 ml. of 0·4 per cent ovomucin dispersion in saline; 1·0 per cent final NaCl concentration.

×———× As above, but 5·0 per cent final NaCl concentration. Inactivation of virus at 65° C. carried out in 5·0 per cent NaCl.

Effect of NaCl on the reduction by active virus of the inhibitory power of ovomucin.

Since the ovomucin is more easily and more uniformly dispersed in 5 per cent NaCl than in 1 per cent, the effect of NaCl on the virus-ovomucin interaction was investigated. It is evident from Fig. 4 that NaCl in 5 per cent concentration markedly inhibits the reduction by active Lee virus of the inhibitory power of ovomucin.

SUMMARY.

Of the various components of hen egg-white, only ovomucin has the power to inhibit haemagglutination by heated type B (Lee) influenza virus.

Ovomucin, as usually prepared, is a protein fraction rather than a single protein. It is always contaminated with lysozyme, in concentrations up to 15 per cent, part of which can be made to crystallize out directly from the ovomucin dispersion.

The inhibitory power of the ovomucin fraction is readily reduced by active influenza virus types A and B, by the receptor destroying enzyme of *Vibrio cholerae* and also by trypsin.

Sodium chloride in 5 per cent concentration inhibits markedly the reduction by active influenza virus of the inhibitory power of the ovomucin fraction.

We are greatly indebted to our colleague, Mr. E. L. French, for his most valuable co-operation in determining by biological assay the lysozyme content of the various ovomucin preparations.

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THE APPLICATION OF PAPER PARTITION CHROMATOGRAPHY TO THE PRODUCTION OF DIPHTHERIA TOXIN; TWO-DIMENSIONAL CHROMATOGRAPHY.

F. V. LINGGOOD AND A. J. WOIWOD.

From the Wellcome Research Laboratories, Beckenham, Kent.

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In a previous communication Linggood and Woiwod (1948) showed that when a substrain, CN 2000, of the Park Williams (P.W.) No. 8 strain of *C. diphtheriae* was grown on a weak casein hydrolysate medium designed for high titre toxin production, nearly all the cystine, aspartic acid, glutamic acid and threonine, detectable by the use of single dimensional paper partition chromatography, were utilized during toxin production, but that there was an increase in the quantity of alanine in the culture filtrate as compared with that in the original medium. Although the most satisfactory separation of amino acids on the chromatogram was obtained using *n*-butanol/acetic acid as solvent, it was not found possible to separate glycine from serine, methionine from valine and leucine from iso-leucine, but a decrease in the intensity of the spots due to these groups could be detected after the growth of the organism. The results in a few instances were confirmed, using phenol and " collidine " as solvents.

In order to investigate more fully the nitrogen metabolism of P.W. 8 and to confirm the results obtained by single dimensional chromatography, it was thought advisable to repeat the work, using two-dimensional paper chromatography to show more conclusively that, for example, the increase in the intensity of the alanine spot was, in fact, due to an increase in the quantity of this amino acid in the culture filtrate and not to the production of a polypeptide moving with a similar R_f value to alanine. Two-dimensional chromatography would also reveal changes in the component amino acids of some of those groups which gave a complex spot on single dimensional chromatograms and enable us to separate glycine from serine and glutamic acid from threonine.

The almost complete utilization of the glutamic acid in the medium during the growth of the organism (Linggood and Woiwod, 1948) led us to study the relationship between the amino acid metabolism and toxin production with this particular substrain of P.W. 8. A comparison was made, therefore, of the toxin production on the basic medium, with and without added glycine or glutamic acid. At the same time, single and two-dimensional chromatograms were made of such media before and after the growth of the organism, but in the case of two-dimensional chromatograms of the media after growth, ultra-filtrates were used in preference to the crude culture filtrates as it was considered that the presence of protein and proteose might complicate the chromatographic picture.

METHODS.

Preparation of culture medium.

Completely hydrolysed casein was prepared, as originally suggested to us by Dr. L. F. Hewitt, by autoclaving Glaxo " Light White Soluble " casein (1 kg.) with pure hydrochloric acid (850 ml.) and water (550 ml.) until the ratio of amino nitrogen, as measured by the method of Pope and Stevens (1939), to total nitrogen was maximal (70 per cent approximately). Under our conditions, 2 hours at 121° C. proved necessary. Ten such lots of hydrolysate were then bulked, diluted out to 40 litres, adjusted to pH 5·5–6·0, treated with sufficient charcoal (Sutcliffe and Speakman No. 5) to give a pale coloured filtrate, which was then diluted to a total nitrogen content of 12·5–13·0 mg./ml. and deferrated according to the method described by Mueller and Miller (1941).

The medium was prepared, as suggested by Holt (1948), by diluting the deferrated casein hydrolysate to a total nitrogen content of 1·0 mg./ml. Cystine, essential nutrients (pimelic acid, nicotinic acid and β-alanine) were then added as described by Mueller and Miller (1941), and the medium, after adjustment to pH 7·4–7·6, was filled out into bottles of rectangular section, 300 ml. per bottle, to give a layer 1·0 cm. deep. The bottles contained varying quantities of 0·1 per cent (w/v) $FeSO_4.7H_2O$ in 1 per cent (v/v) hydrochloric acid to give a range of added Fe · · · from 0·02 μg./ml. to 0·20 μg./ml. of medium. This medium, referred to in the text as Medium A, had a total nitrogen content of 1·0 mg./ml. and a sodium chloride content of 0·45 per cent (w/v).

Toxin production on Medium A was compared with that obtained when such a medium was fortified, firstly with 0·15 per cent (w/v) of glycine (Medium B) and, secondly, with 0·29 per cent (w/v) of glutamic acid (Medium C). Both the latter media had a total nitrogen content of 1·3 mg./ml.

Bottles containing all three media were sterilized for 10 minutes at 115·5° C. When cool, 9·0 ml. of the maltose-$CaCl_2$ mixture, prepared as described by Mueller and Miller (1941), was then added to each bottle. This was the amount of maltose which had been found optimal for toxin production on these media under our conditions.

Toxin production.

The " Toronto " strain of the Park Williams No. 8 strain of *C. diphtheriae* was used throughout this work, the particular substrain employed being labelled CN 2000 in the Wellcome Collection. This organism was grown concurrently on Medium A, Medium B and Medium C, the number of bottles being so arranged that there were at least four bottles at every level of added Fe · · · for all three culture media. After 8 days at 34° C. the bottles were removed from the incubator and toluolised. The four bottles at each level of added Fe · · · were then bulked, filtered, and toxin estimations done on the culture filtrates by the flocculation method (Ramon, 1922 ; Glenny and Okell, 1924). With each of the three culture media, that filtrate showing the highest titre toxin was then used for paper chromatography.

Ultrafiltration.

For two-dimensional chromatography the culture filtrates used in this work were freed from protein by ultrafiltration through Berkefeld type candles coated

with 9 per cent (w/v) collodion in glacial acetic acid (I.C.I.). The liquid passing through such membranes was shown to be free from protein by the addition of one-tenth volume of 50 per cent (w/v) trichloroacetic acid and also to contain no toxin as measured by the flocculation test after blending with a quick flocculating toxin (Glenny, Pope, Waddington and Wallace, 1926).

Paper partition chromatography.

Single and two-dimensional paper chromatograms were made of culture media, culture filtrates and ultrafiltrates of the latter, No. 4 Whatman paper being used throughout. For single dimensional chromatography *n*-butanol-acetic acid was used as solvent (Partridge, 1948), for two-dimensional work the same solvent was used initially and then phenol as solvent for running in a direction at right angles to the first. This combination of solvents did not allow us to separate leucine from iso-leucine or valine from methionine, but gave a good separation of those amino acids which single dimensional chromatography had shown to be of most interest.

It was found that the addition of 0·1 per cent (v/v) " collidine " to the solution of ninhydrin in chloroform (0·1 per cent (w/v)) used for spraying the papers gave a better differentiation of colour with the spots of amino acids. With such a reagent glycine gave a reddish brown and serine a blue colour and so enabled us to detect changes in the amounts of these two amino acids even on single dimensional chromatograms, on which they both moved with nearly the same R_f value.

All the two-dimensional chromatograms shown in this paper were run, as far as possible, under identical conditions in a constant temperature room and in many instances chromatograms which were to be compared were run in the same tank at the same time. A constant volume containing approximately 50μg. of nitrogen on a spot 1/5 inch in diameter was used for each two-dimensional chromatogram.

RESULTS.

In Fig. 2 is given the photograph of the most important part of the two-dimensional chromatogram of the basic medium, i.e. the casein hydrolysate diluted out to a total nitrogen figure of 1·0 mg./ml. with cystine and the essential nutrients added, but containing no added glycine or glutamic acid (Medium A). In the photograph each spot has been labelled with the name of the amino acid which it represented, the position taken up by each amino acid having been previously determined by running under similar conditions a mixture of pure amino acids. It can be seen from this photograph that the strongest spot was due to glutamic acid, as would be expected from the fact that casein contains more glutamic acid than any other amino acid (Block and Bolling, 1945). Likewise, serine and the basic amino acids gave intense spots. In contrast, the alanine present gave quite a weak spot and glycine, of which casein contains only 0·5 per cent (Block and Bolling, 1945), was just visible. The behaviour of aspartic acid on our two-dimensional chromatograms was rather abnormal for it gave two spots close together. As both these spots became more intense when aspartic acid was added to the medium (Fig. 8) it was concluded that, under our conditions, for reasons unknown, aspartic acid ran as two spots. Similar behaviour with aspartic acid on two-dimensional chromatograms has also been observed by Dent (personal communication).

Fig. 4 gives the photograph of the most important part of the chromatogram of the basic medium with added glycine (Medium B); Fig. 6 that of the basic medium with added glutamic acid (Medium C); Fig. 8 that of the basic medium with both glutamic and aspartic acids added.

Before preparing two-dimensional chromatograms of the media after growth, those culture filtrates showing the highest titre toxin on all three media were run at one time on a single sheet of paper using *n*-butanol-acetic acid as solvent, each filtrate in parallel with its corresponding culture medium.

A photograph of the single dimensional chromatogram thus obtained is given in Fig. 1, where it will be seen that with all three culture media there had been a considerable utilization of aspartic acid and an almost complete removal of glutamic acid during the growth of the organism. An increase in the intensity of the alanine spot was evident with culture filtrates from Medium B and Medium C, but not with Medium A. The disappearance, after growth, of the glycine-serine spot, except with Medium B where glycine had been added to the medium, suggested that serine might play an important part in toxin production. Although not showing in the photograph, it was noted from the original chromatogram which had been sprayed with ninhydrin in chloroform (0·1 per cent (w/v)) containing 0·1 per cent (v/v) "collidine" that the serine had disappeared during the growth of the organism with Medium B, for there was none of the blue colour of serine on the glycine-serine spot on the chromatogram of the culture filtrate, but only the reddish brown colour of glycine, whereas both colours were clearly visible on the same spot of the chromatogram of Medium B. Other points of interest which had not been noticed before were the increase in the intensities of the tyrosine and phenylalanine spots after the growth of the organism. As these observations might have been due, however, to the presence of polypeptides moving with the same R_f values as tyrosine and phenylalanine these points will be investigated more fully later.

To ensure, however, that the interpretation of such single dimensional chromatograms was correct, two-dimensional chromatograms were then made of the ultrafiltrate from each culture filtrate. Photographs of the most important part of the chromatogram of Medium A and that of the corresponding ultrafiltrate are shown in Fig. 2 and Fig. 3. These photographs confirm the results obtained with this medium by single dimensional chromatography, as shown in Fig. 1.

Likewise, Fig. 4 and Fig. 5 give photographs of the chromatograms of Medium B, which was the medium studied in our original paper (Linggood and Woiwod, 1948), and its corresponding ultrafiltrate, and present a similar picture to that for Medium A, except that the added glycine had not been completely utilized and that there had been an increase in the amount of alanine in the ultrafiltrate compared with that in the original culture medium. Thus the observations reported previously (Linggood and Woiwod, 1948) have been confirmed by two-dimensional chromatography.

Similarly, Fig. 7 gives the photograph of the ultrafiltrate of the culture filtrate from the culture medium to which additional glutamic acid had been added (Medium C) and should be compared, therefore, with Fig. 6. It will be seen that the whole of this not inconsiderable amount of added glutamic acid had disappeared completely during toxin production and that there had been a complete elimination, as far as could be detected by paper chromatography, of the slower

moving amino acids in the solvents used with the exception of the basic amino acids and alanine, the latter showing an increase after growth.

The relative toxin production obtained on these three culture media is given in Table I. All Lf values quoted are those obtained with the optimal amount

TABLE I.—*Showing the Relative Toxin Production with CN 2000 on Casein Hydrolysate Media with and without Added Glycine or Glutamic Acid.*

Number.	Medium A Lf units/ml.	Medium B Lf units/ml.	Medium C Lf units/ml.
L 561	59	65	..
L 566	60	61	89
L 570	67	..	100
L 572	61	61	..
L 573	65	66	..
L 574	66	61	93
L 576	65	72	104
L 582	..	82	104
L 587	..	75	97
L 588	..	80	92
L 595	70	76	96
L 596	..	90	112
L 602	86	89	116
L 605	62	61	96
L 607	64	80	86
L 609	82	103	114
L 611	75	92	104
L 620	70	86	106

Medium A: Casein hydrolysate medium (total nitrogen = 1·0 mg./ml.).

Medium B: Casein hydrolysate medium fortified with 0·15 per cent glycine (total nitrogen = 1·3 mg./ml.).

Medium C: Casein hydrolysate medium fortified with 0·29 per cent glutamic acid (total nitrogen = 1·3 mg./ml.).

of Fe · · · added to the medium. From this table it will be seen that the lowest toxin production was with Medium A, while Medium B gave sometimes, but not invariably, appreciably higher toxin titres. With Medium C, however, high titre toxin (100 Lf units/ml. ± 10 Lf units/ml.) was obtained more consistently than with Medium B. It was noted, too, that with our strain of P.W. 8 the growth of the organism was usually better on Medium C than on Medium A or on Medium B, and since this work was done, bulk batches of toxin have been produced using Medium C having titres from 85–110 Lf units/ml. It was found that the pH of the culture during growth was maintained nearer to pH 7·0–7·5 with Medium C than with either Medium A or B, both the latter having a pH at the end of growth usually between pH 6·0–6·5. When, however, less maltose was added to either Medium A or Medium B in an attempt to obtain a higher pH during growth, lower titre toxins were produced.

DISCUSSION.

The results obtained indicate quite clearly the value of paper partition chromatography in a study of diphtheria toxin production on weak casein hydrolysate

medium, for not only has this technique served the practical purpose of increasing the toxin production with our strain of P.W. 8, but it is felt that the results are of considerable theoretical interest, pointing, among other things, to the importance of glutamic acid in the nitrogen metabolism of *C. diphtheriae* as has already been demonstrated for other organisms (Gale, 1948).

It is appreciated that small differences of the order of 5 per cent in the quantity of any amino acid on a single dimensional chromatogram cannot be detected by inspection, but it has been shown by Woiwod (unpublished observations) that with glycine and glutamic acid differences of 20 per cent are quite obvious by inspection, especially when the materials under examination are run side by side on the same sheet of paper.

It is felt, therefore, that any difference in the chromatogram of the medium before and after the growth of the organism does mean that an appreciable change has occurred in the amino acid content of the medium during toxin production. On the other hand, because no difference can be detected in the intensity of the spot of a certain amino acid on the chromatogram of the medium before and after growth, it does not mean that that amino acid has not been utilized at all during growth, but only that the change, if any, taking place is not sufficient to be seen by inspection of the chromatogram. It is intended, at a later date, to obtain, by the method developed by Woiwod (1948*a*, 1948*b*) for the micro estimation of amino nitrogen, quantitative figures for the changes which occur in the content of various amino acids, separated from the medium by paper chromatography, before and after toxin production.

Nitrogen estimations on the washed organisms, at the end of the growth period on Medium C, at the iron optimum for toxin production, gave a figure of 100 mg. of bacterial nitrogen per culture bottle of 300 ml. of medium. The nitrogen content of the uninoculated medium was 1·3 mg./ml., i.e. 390 mg. per bottle. Hence the nitrogen content of the washed organisms represents 26 per cent approximately of the nitrogen content of the original medium. Similarly, estimations of the protein nitrogen content of the culture filtrate gave a figure of 35 mg. of protein per culture bottle, i.e. 9 per cent approximately of the nitrogen content of the original medium appears in the culture filtrate as protein nitrogen.

EXPLANATION OF PLATES.

FIG. 1.—Single dimensional chromatograms of Medium A, Medium B and Medium C run in parallel with their corresponsing culture filtrates with *n*-butanol-acetic acid as solvent. Key to amino acids in this chromatogram : (*b*) basic amino acids, i.e. lysine, arginine and histidine ; (*c*) aspartic acid ; (*d*) glycine and serine group ; (*f*) glutamic acid and threonine group ; (*h*) alanine ; (*k*) proline ; (*l*) tyrosine ; (*n*) valine and methionine group ; (*o*) phenylalanine ; (*p*) leucine and isoleucine group.

FIG. 2.—Two-dimensional chromatogram of Medium A.

FIG. 3.—Two-dimensional chromatogram of ultrafiltrate of culture filtrate from Medium A.

FIG. 4.—Two-dimensional chromatogram of Medium B.

FIG. 5.—Two-dimensional chromatogram of ultrafiltrate of culture filtrate from Medium B.

FIG. 6.—Two-dimensional chromatogram of Medium C.

FIG. 7.—Two-dimensional chromatogram of ultrafiltrate of culture filtrate from Medium C.

FIG. 8.—Two-dimensional chromatogram of basic medium with both glutamic and aspartic acids added.

FIG. 9.—Complete two-dimensional chromatogram of hydrolysate of washed organisms of P.W. 8.

FIG. 10.—Complete two-dimensional chromatogram of hydrolysate of protein present in culture filtrate from Medium C (200 μg. N taken for analysis instead of 50 μg. N as with other two-dimensional chromatograms).

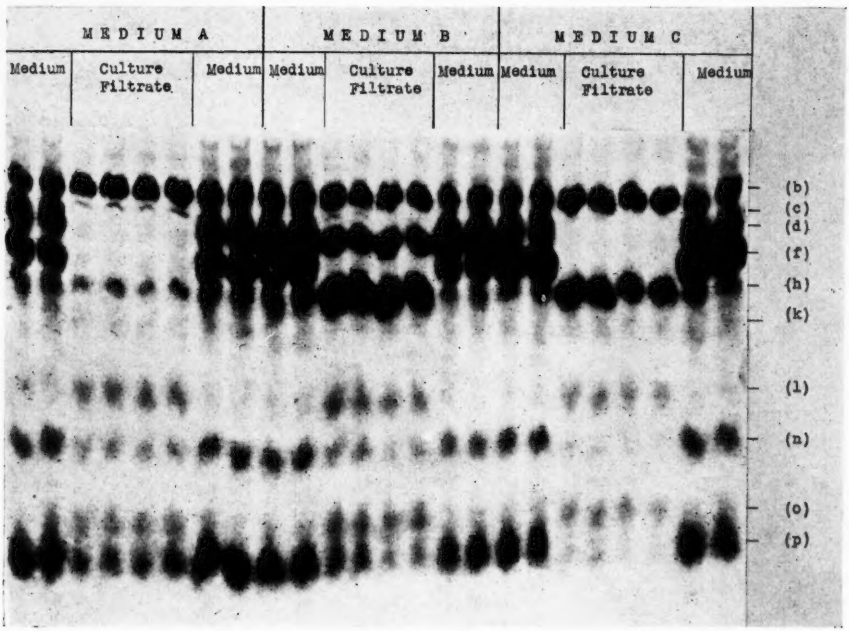
MEDIUM A
MEDIUM B
MEDIUM C
Medium
Culture Filtrate
Medium
Medium
Culture Filtrate
Medium
Medium
Culture Filtrate
Medium
(b)
(c)
(d)
(f)
(h)
(k)
(l)
(n)
(o)
(p)

FIG. 1.

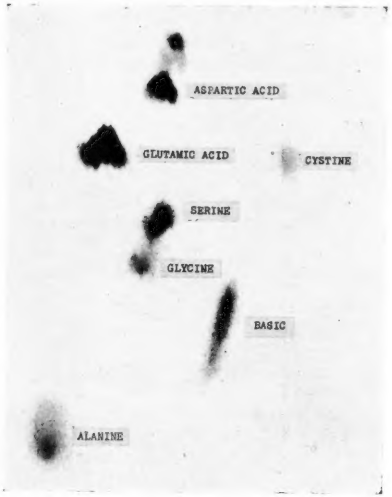

FIG. 2.

FIG. 3.

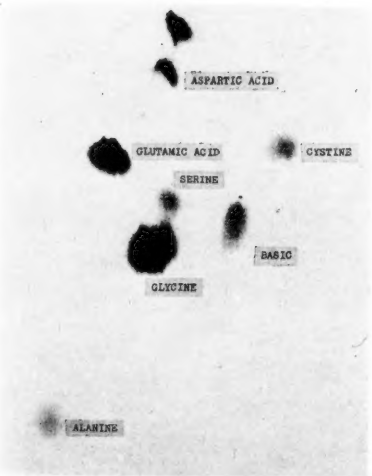

FIG. 4.

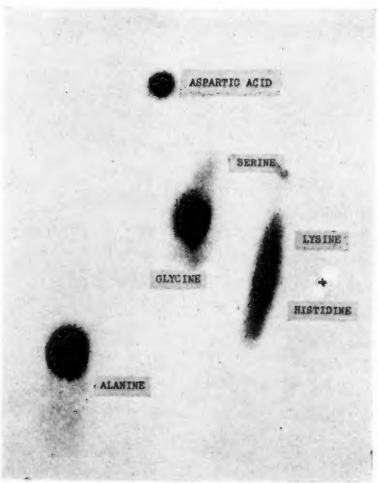

FIG. 5.

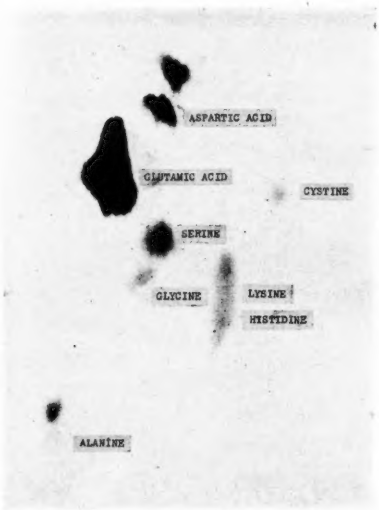

FIG. 6.

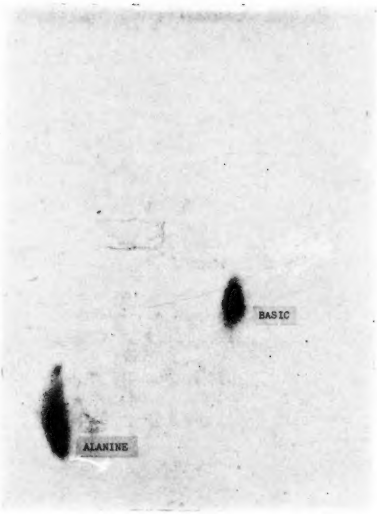

FIG. 7.

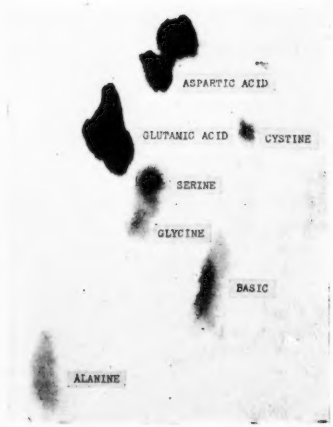

FIG. 8.

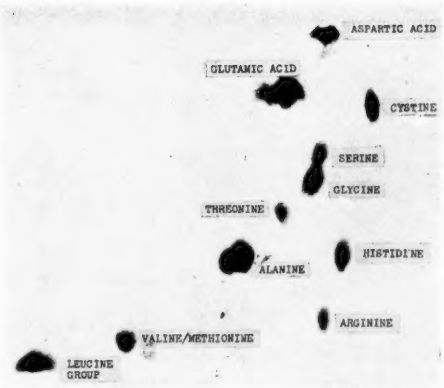

FIG. 9.

ASPARTIC ACID
GLUTAMIC ACID
SERINE
LYSINE
GLYCINE
HISTIDINE
THREONINE
TYROSINE
ALANINE
ARGININE
VALINE/METHIONINE
LEUCINE GROUP

FIG. 10.

The nitrogen content of the added glutamic acid in Medium C is 0·3 mg./ml., i.e. 90 mg. per culture bottle of 300 ml. of medium. This figure does not, however, represent the whole of the glutamic acid nitrogen in the completed medium for there is some glutamic acid in the casein hydrolysate which constitutes the basic medium. From the figures given in Block and Bolling (1945) for the amino acid composition of casein, it can be calculated that in 300 ml. of Medium C there is an additional 40 mg. approximately of glutamic acid nitrogen due to the casein hydrolysate, making a total of 130 mg. of nitrogen due to glutamic acid in one bottle of Medium C.

It will be seen, therefore, if all the glutamic acid disappears from the medium during the growth of the organism, as would seem highly probable from our chromatograms, the nitrogen content of the glutamic acid which disappears is of the same order as the combined nitrogen content of the organisms and protein excreted during growth. Fig. 9 gives a photograph of the two-dimensional chromatogram obtained by running the hydrolysate of washed organisms and Fig. 10 that by running the hydrolysate of the protein in the culture filtrate. It will be seen from both these photographs that glutamic acid is by no means the major amino acid constituent present in either of these hydrolysates. It is probable, therefore, that much of the glutamic acid which disappears from the medium during the growth of the organism is not used solely as a " building brick " in the production of protein, but is changed into other substances by the enzymes of the growing organism. It is quite possible that among such enzymes is a transaminase as originally suggested by us (Linggood and Woiwod, 1948) to account for the increased quantity of alanine in the culture filtrates. Experiments are now in progress to see whether it is possible to detect the presence of a transaminase in washed suspensions of the organism which is capable of converting glutamic acid into alanine in the presence of a keto acid (Darling, 1945).

SUMMARY.

Single and two-dimensional paper chromatograms have been made of three casein hydrolysate culture media before and after the growth of our strain of the Park Williams No. 8 strain of *C. diphtheriae*. These three culture media were designed for high titre toxin production; Medium A consisted of deferrated casein hydrolysate diluted to a total nitrogen figure of 1·0 mg./ml. with cystine and essential nutrients added, while Medium B had an additional 0·15 per cent glycine and Medium C an additional 0·29 per cent glutamic acid added. Toxin production was lowest with Medium A, usually somewhat better with Medium B, but Medium C gave more consistently high toxin titres of 100 Lf units/ml. $\pm$ 10 Lf units/ml. and showed the most marked changes in the chromatograms of the medium before and after growth, all the cystine, aspartic acid, serine and glutamic acid, detectable by paper partition chromatography being utilized during toxin production, while the amount of alanine in the culture filtrate increased as compared with that in the culture medium before growth.

Similar changes were noted with Medium B except that the added glycine was not completely utilized. Thus our original observations (Linggood and Woiwod, 1948) have been confirmed by two-dimensional chromatography. Less marked changes were observed with Medium A.

Our thanks are due to Dr. C. G. Pope and Dr. A. E. Francis for criticism of the manuscript, and to Mr. E. A. Jones and Mr. E. E. Hitchcock for the photography.

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LATEROSPORIN A AND LATEROSPORIN B, ANTIBIOTICS PRODUCED BY *B. LATEROSPORUS*.

ELLA M. BARNES.

From the Sir William Dunn School of Pathology, Oxford.

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A considerable amount of work has now been done on antibacterial substances produced by sporing organisms of the genus *Bacillus*. This work is summarized by Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey (1949).

Using the cross streak technique, Gilliver (1947, unpublished) showed that *B. laterosporus* (Gibson, No. 1066), when grown on the surface of a Lemco Agar plate produced a diffusible substance which inhibited the growth of a number of organisms. An account is given below of two antibiotics that have been called laterosporin A and B produced by this strain of *B. laterosporus*.

EXPERIMENTAL.

Assay and unit of activity.

Activity was assayed by the cylinder plate method described by Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings (1941). Laboratory strains of *Myco. phlei*, *C. xerosis* and *Staph. aureus* (H strain) were used as test organisms. A dry preparation of the hydrochloride of laterosporin A was adopted as an arbitrary standard—1 mg. being taken to contain 10 units. One unit per ml. was found to produce inhibition zones of 14–18 mm. under the conditions of the assay used.

The curve relating the concentration of laterosporin A to the diameter of the zone of inhibition flattened considerably at concentrations above 1 unit per ml., and for all routine assays the test solutions were diluted to give values below 1 unit per ml. It was also found necessary to make a standard curve for each assay plate, and assays were carried out in duplicate against at least two of the test organisms.

Medium and conditions of culture.

For routine antibiotic production the organism was grown in the following medium—glucose 0·5 per cent, yeast extract (centrifuged water extract of dried autolysed yeast) contributing 0·5 per cent soluble solids, KH_2PO_4 0·05 per cent, K_2HPO_4 0·05 per cent, $(NH_4)_2SO_4$ 0·5 per cent, made up with tap water, pH6·6. The glucose and yeast extract, diluted with tap water, were autoclaved at 1 atmosphere for half an hour in 1 litre quantities in glass culture vessels, the medium having a depth of 40 mm. A solution of 20 per cent $(NH_4)_2SO_4$, 2 per cent KH_2PO_4, and 2 per cent K_2HPO_4, was autoclaved separately and 25 ml. added to each vessel before inoculating with 0·5 per cent of a 2-day culture of the organism in Lemco broth.

The vessels were incubated for 3–4 days at 37° C. The organism grew throughout the medium and formed a fairly thick, but easily dispersed pellicle which partly covered the surface. Spores were seldom formed. Approximately 1 unit per ml. was produced. The final pH of the medium was 5·8–6·0. When grown in 20 mm. layers the activity per ml. was slightly higher, but for the routine preparation of 50 or 100 litre batches it was easier to work with 1 litre of medium in a vessel.

Before the medium described above was evolved a number of experiments were carried out in order to obtain as simple a medium as possible both for preparation and extraction purposes.

Phosphates.—The addition of phosphate was found to be necessary for optimal growth and activity. However, 0·05 per cent KH_2PO_4 with 0·05 per cent K_2HPO_4 gave a higher activity than 0·1 per cent KH_2PO_4 with 0·1 per cent K_2HPO_4. If the phosphates were autoclaved with the yeast extract and the glucose, a heavy precipitate came down, probably due to reaction with the Ca and Mg ions in the tap water. Growth and production of antibiotic was poor in this medium.

Glucose.—In early experiments the glucose was sterilized either by filtration or by separate autoclaving. When the glucose was autoclaved with the yeast extract at pH 6·6 growth and production of antibiotic were delayed about 24 hours, but the maximum growth and production of the antibiotic were not significantly different from those in controls in which the glucose was added separately. Autoclaving the yeast extract with the glucose at higher pH values (7·0 and 7·5) delayed growth and production of antibiotic still further. Glucose was found to be necessary for optimal antibiotic production, but no significant difference could be detected between the potencies of the active liquors produced with 0·5 per cent and 2 per cent glucose.

Nitrogen.—Increasing the yeast extract to 1 per cent in the medium did not improve growth or activity. The yeast extract could be replaced by 1 per cent Evan's peptone without loss of activity. Stansly, Schlosser, Ananenko and Cook (1948) reported that the addition of 2 per cent $(NH_4)_2SO_4$ to a Difco yeast extract,

glucose and inorganic salts medium, greatly increased the yield of polymyxin without an increase in the growth of the organism as compared with the growth in the control medium with no $(NH_4)_2SO_4$. To determine the effect on the production of laterosporin, sterile solutions of $(NH_4)_2SO_4$ were added to the medium to give a final concentration of 0·5 per cent and 1 per cent. The addition of 0·5 per cent $(NH_4)_2SO_4$ doubled the amount of antibiotic produced. A slightly higher activity was given by 1 per cent than by 0·5 per cent. Although there did not appear to be any significant difference in total growth, the organism grew faster in the presence of $(NH_4)_2SO_4$ and the maximum activity was reached earlier. Autoclaving $(NH_4)_2SO_4$ with the yeast extract and glucose resulted in slightly lower activity.

Extraction.

The culture fluid containing approximately 1 unit per ml. of activity was acidified to pH 2·0, and was either allowed to settle overnight or was centrifuged to remove cells and debris. The pH of the supernatant liquor was raised to 6·5–7·0 and the active material adsorbed on to 0·5 per cent charcoal (Farnell, Grade 14, neutral). The charcoal was eluted by shaking vigorously with a mixture of 2 parts butanol and 3 parts N/5 HCl, the quantity used being 1/20th of the volume of the original culture fluid. Almost all the activity eluted from the charcoal was concentrated in the butanol layer, not more than 2 units per ml. remaining in the acid layer. The method of elution from charcoal with a two phase system of butanol and HCl was first used successfully with Ayfivin by Arriagada, Abraham, Sharp, Savage, Sharp and Heatley (unpublished, 1949). The acid layer was discarded. The laterosporin was recovered from the butanol layer by shaking with 2 volumes of ether and 1/5th volume of water. The water concentrate was adjusted to pH 6·8–7·0 and filtered.

The concentrate was cooled and laterosporin picrate precipitated by the addition of an excess of saturated aqueous picric acid solution. The picrate was dried in a vacuum desiccator, ground to a fine powder, and converted to the hydrochloride by suspension in ethyl alcohol containing 5 per cent acid. Not all the picrate went into solution ; the insoluble portion was centrifuged off and resuspended in more acid alcohol. This process was repeated until the colour of the alcoholic solution remained unchanged.

The acid alcohol extracts were combined and treated with dry ether until no more precipitate came down. The precipitate was washed in ether and dried *in vacuo*. The light brown powder so formed had an activity of about 10·5 units per mg. and was called laterosporin A. The remaining material, which was insoluble in acid alcohol, was washed with ether and dried. It was a yellow powder having an activity of about 12·6 units per mg. and was called laterosporin B. It could be freed from any insoluble material by dissolving in N/100 HCl containing 0·5 per cent NaCl and extracting with butanol. The active material was precipitated from the butanol layer by the addition of ether and dried to form a cream coloured powder. The activity of this material was approximately 11·6 units per mg., so did not differ significantly from the original laterosporin B.

About 25–30 per cent of the active substances were usually recovered by the above process, but yields up to 50 per cent have been obtained. The ratio of laterosporin A to laterosporin B varied, but the average ratio was 1 : 1·5.

Chemical properties.

No evidence is yet available of the purity of the preparations of laterosporin A or B.

Laterosporin A.—This appeared to be of a peptide nature. After hydrolysis with 5N HCl in a sealed tube at 110° C., a preliminary analysis by paper chromatography indicated that it contained a number of different amino acids.

Laterosporin A was not destroyed by trypsin. A solution in water of 1 unit per ml. lost no significant activity when heated at 100° C. for half an hour between pH's 2·0 and 8·0.

Moderate solubility at pH 2·0 decreased with increasing pH to 9·0, where it was only sparingly soluble. Partition experiments with equal volumes of butanol and an aqueous phase showed that in N/100 HCl only about 25 per cent of the activity entered the butanol layer, but that with 0·05 per cent NaCl present 66 per cent went into the butanol and with 0·5 per cent NaCl all the activity was extracted into the butanol layer.

The substance was not extracted from water at any pH by ether, chloroform, or amyl acetate.

Laterosporin A dialysed through a cellophan membrane.

Laterosporin B.—The few observations made indicated a very close resemblance of laterosporin A to laterosporin B. Laterosporin B was soluble in water and the activity of the hydrochloride against the standard test organisms was of a similar order to that of laterosporin A. Laterosporin B also showed similar properties with regard to partition into butanol in the presence of NaCl. Preliminary examination of the acid hydrolysed product by paper chromatography did not reveal any differences in the chromatogram compared with that of laterosporin A.

Laterosporin B was, however, insoluble in absolute ethyl alcohol in contrast to laterosporin A, which was soluble.

Antibacterial activity.

The sensitivity of a number of organisms to laterosporin A and B was determined by a serial dilution method in broth. Solutions of the hydrochlorides were sterilized either by filtration through a sintered glass bacteriological filter or by heating in N/20 HCl at 97° C. for half an hour. The solutions were diluted in twofold series and the dilutions added to nutrient broth. Ten per cent of serum was present for the streptococcus and *C. diphtheriae*. The tubes were inoculated with an overnight broth culture undiluted or diluted up to 1000 times. After overnight incubation the end-point was read as that concentration of laterosporin which completely inhibited growth. Further incubation for up to three days did not increase the inhibitory concentration more than two-fold. The results obtained are shown in Table I. No significant difference could be detected in the antibacterial activity of laterosporin A and B.

Assays against *Myco. tuberculosis* human type, strain P.N., in Dubos medium showed partial inhibition at 1 unit per ml. with both laterosporin A and B after incubation for 7 days. This test was performed by Dr. A. Q. Wells.

A decrease in the size of inoculum with *Staph. aureus* (H strain) from an undiluted broth culture to a 1/1000 dilution decreased the inhibitory concentration fourfold at 16 hours and twofold at 48 hours.

The addition of 50 per cent horse serum to solutions of laterosporin A and B reduced the activity to half its original value. No further loss of activity occurred after incubating the solutions for 4 hours at 37° C.

TABLE I.

Species of Organism.	Concentration of laterosporin in units/ml. which totally inhibited growth in 16 hours.	
	Laterosporin A.	Laterosporin B.
Staphylococcus aureus (H strain)	0·01	0·01
Streptococcus pyogenes Group A	0·12	0·12
,, ,,	0·12	0·12
,, ,,	0·06	0·03
Corynebacterium diphtheriae var. *gravis*	0·12	0·12
,, *xerosis*	0·005	0·003
Mycobacterium phlei	0·0008	0·0004
,, *smegmatis*	10·0	10·0
,, *tuberculosis* human type	*1·0 (partial)	*1·0 (partial)
Salmonella typhi	0·6	0·6
,, *enteritidis* (Gaertner)	1·2	2·5
Bacterium coli	0·6	0·6
Pseudomonas pyocyanea	5·0	10·0

* Grown in Dubos medium for 7 days.

SUMMARY.

An antibiotic produced by a strain of *B. laterosporus* has been investigated. A method is given for the production, extraction and partial purification of the active material. Some chemical properties are described and evidence presented for the existence of two separate though closely related substances which have been named laterosporin A and laterosporin B. Antibacterial activity has been demonstrated against several groups of organisms.

I am indebted to Prof. Sir H. W. Florey and Dr. E. P. Abraham for advice and encouragement, and to Dr. M. A. Jennings for supervising the serial dilution tests and for reading the manuscript, to Miss M. Bond for technical assistance with the dilution tests, and Miss J. Davidson for general technical assistance.

The work has been carried out while holding a personal grant from the Medical Research Council who have also contributed towards the expenses of materials and technical assistance.

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ACUTE EXPERIMENTAL CALCIUM CHLORIDE ACIDOSIS.

A. D. TELFORD GOVAN AND J. PARKES.

From the Research Department, The Glasgow Royal Maternity and Women's Hospital.

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In a previous publication (Govan and Parkes, 1946) it was shown that the administration of ammonium chloride to rabbits caused acidosis and ultimately death of the animals. Widespread necrosis of the renal tubules occurred, suggesting that death might be due to uraemia. In certain respects, however, the biochemical changes were atypical, and did not fit into the syndrome usually shown in acute experimental nephrosis. In almost all cases of experimental nephrosis recorded there has been a marked reduction in the output of fluid and a retention of urea. This has been reported in the case of poisoning by uranium (Frothingham, Fitz, Folin and Denis, 1931), tartrates (Underhill, Wells and Goldschmidt, 1913), oxalates (Dunn, Haworth and Jones, 1924), uric acid (Dunn and Polson, 1926), and by mercury (McNider, 1918). After administration of ammonium chloride the blood urea rose, but the highest recording was 173 mg. per cent, and the urinary output of fluid and nitrogen remained high. These results resemble those found by Folin, Karsner and Denis (1912) in animals poisoned by chromates and cantharidin. It was suggested at the time that the relatively low blood urea values obtained after ammonium chloride might be due to the maintenance of fluid output, since Dunn and Jones (1925) have shown that intra-peritoneal injections of sodium chloride increased the fluid output and decreased the blood urea in rabbits rendered nephritic by oxalates. It is interesting to note, however, that Underhill, Wells and Goldschmidt found that the urea output was nil, although fluid was being excreted by animals poisoned by tartrates.

That there is some connection between acidosis and renal damage is suggested by the reports of diminished plasma CO_2 combining power after administration of proved nephritic agents, such as uranium (Goto, 1917), cantharidin, arsenic, diphtheria, chromates (Goto, 1918*a*, 1918*b*) and mercury (McNider, 1918 ; Ogilvie, 1932). Goto (1918*a*, 1918*b*) found retention of chlorides and urea as well as acidosis in these cases, and the administration of sodium bicarbonate by mouth lessened the severity of the changes.

It seemed worth while therefore to pursue the study of severe acute acidosis with the hope of eliciting further information regarding the cause of death of these animals and the relationship between acidosis and kidney function. In the experiments to be described it was decided to use calcium chloride, since in our previous work it was impossible to eliminate the ammonium ion as a possible factor in the cause of death.

METHODS.

Experimental.

Preliminary experimentation showed that doses of ammonium chloride and calcium chloride containing equivalent amounts of chlorine produced widely varying results. It required a relatively greater dose of calcium chloride to produce changes similar to those obtained with ammonium chloride. With the ammonium salt toxic changes resulting in death of the rabbit within four days were produced with a dose of 0·75 g. per kg., but it required a dose in excess of 2 g. per kg. of calcium chloride to do this.

Twelve rabbits were used in the experiment. These were housed in urine cages. Food consisted of moist bran and oats fed in bird-cage feeding boxes. Urines were collected daily for the estimation of urea, chlorides, phosphates, ammonia and pH. Routine tests for albumen were carried out. The animals were maintained on this simple diet for a week, blood being withdrawn every second day for analysis.

At the end of the week calcium chloride in a 10 per cent solution was administered daily by stomach-tube. Two animals received 1·5 g. per kg., two received 1·8 g. per kg., two received 2·2 g. per kg. and six received 2·5 g. per kg. Blood and urine examinations were carried out as above.

Chemical.

Urinary and blood urea values were estimated by the hypobromite and urease methods respectively. The usual Volhard technique was employed for chlorides. Kuttner and Lichtenstein's (1930) method was used for the estimation of phosphates. Ammonia levels were found by the aeration method. The apparatus used was that described by Van Slyke and Cullen (1916). It consisted of three corked Pyrex boiling tubes connected in series by glass and rubber tubing so that air could be sucked through them by means of a water pump attached to Tube 3, the inlet being at Tube 1. Tube 1 contained 5 per cent sulphuric acid in order that traces of ammonia in the atmosphere could be removed as the air bubbled through the acid before reaching Tube 2 containing the blood and reagents. Tube 3 contained 25 c.c. of 0·02 N hydrochloric acid. Blood was then collected under liquid paraffin. Five ml. of blood were placed in Tube 2. One drop of caprylic alcohol and 4 or 5 g. of potassium carbonate were added to the tube, which was corked and then shaken gently to mix the contents. A slow current of air was then drawn through the tubes for 2 or 3 minutes, followed by a rapid current for 15 minutes. At the end of this time the excess acid in Tube 3 was titrated with 0·02 N sodium hydroxide, and the necessary calculations made from the titration figure. Blank determinations on the reagents were made on occasion to eliminate the possibility of ammonia being present as an impurity. Serum calcium was determined, using the method of Kramer and Tisdall (1921) as modified by Tisdall (1923).

Histological.

Formol saline was used as a routine fixative for paraffin and frozen sections. Representative specimens for the demonstration of glycogen and phosphatase were fixed in alcohol. Sections were stained by haematoxylin and eosin, Masson's trichrome method, Dunn's aniline blue Orange G method, Gomori's method for alkaline phosphatase, Best's carmine stain for glycogen and Sudan IV.

RESULTS.

During the first week prior to calcium chloride administration the animals appeared to be healthy. Urinary volume was high at the beginning. In six animals it fell slightly during the first two or three days of the new diet, but rose or remained steady thereafter. Urea output was roughly parallel to water excretion, although the curve was always much flatter and changes in the curve were delayed. The excretion of phosphate rose steeply, while chloride output fell. Urinary pH fell gradually; blood analysis showed that urea, chlorides, lipoids, ammonia and pH were all within normal limits. The CO_2 combining power of the blood was lower than usual, but still within normal limits. This appears to be due to the absence of greens in the diet. This feature was noted in our previous experiments with ammonium chloride. Serum calcium and phosphorus were higher than usual, but the Ca:P ratio was normal.

In their reaction to calcium chloride the animals formed two distinct groups comprising (*a*) all animals receiving 1·5 to 1·8 g. per kg. plus one animal receiving 2·2 g. per kg.; and (*b*) all animals receiving 2·5 g. per kg. plus one animal receiving 2·2 g. per kg.

Group 1.

Five animals—four receiving 1·5 to 1·8 g. calcium chloride per kg. and one receiving 2·2 g. per kg.

All of the animals in this group were killed on the 5th day of the experimental period. During the first four days of this period the animals showed no signs of toxaemia, ate their food and appeared to be healthy in every way.

During the first 24 hours the urinary volume diminished. Urea excretion ran almost parallel with the output of water, but although the volume of urine was increased at the 48-hour period there was a 24-hours' delay in the rise of urea excretion. Both were increased at 72 hours, but at 96 hours the urea output tended to fall although water excretion remained high. The excretion of phosphates increased slightly during the first 24 hours, but fell moderately although progressively thereafter. Chlorides, naturally, showed a steeply rising curve. Urinary pH fell rapidly and remained round 5. Albuminuria occurred in all cases as soon as the pH fell below 6.

The most marked change in blood chemistry was a fall in CO_2 combining power. Prior to the experimental period the average normal CO_2 combining power was approximately 40 volumes per cent. After calcium chloride the average was 13·5 volumes per cent. At the same time the plasma pH was reduced slightly. Urea was slightly increased; blood cholesterol was almost doubled, and chlorides increased by 25 per cent. Calcium and phosphorus were slightly increased and the blood lipoids remained normal. Table I shows a comparison of average values before and after administration of calcium chloride.

TABLE I.

	Blood urea, mg./100 ml.	CO_2 c.p., vol./100 ml.	pH.	Cl', mg./100 ml.	Serum Ca. mg./100 ml.	Serum P. mg./100 ml.	Cholesterol, mg./100 ml.	Lipoids, mg./100 ml.	Ammonia, mg./100 ml.
Before	42·1	40	7·19	594	12·0	3·76	43·6	0·14	0
After	57·7	13·5	6·8	742	13·3	4·25	70	0·13	0

The graph in Fig. 1 shows the detailed changes in Animal 2, which is representative of the group. All of these animals were killed at the beginning of the 5th day of the experimental period by a sharp blow on the back of the neck. At post-mortem the appearances were similar in all cases. The lungs were emphysematous, but showed a few congested areas of collapse. No change could be

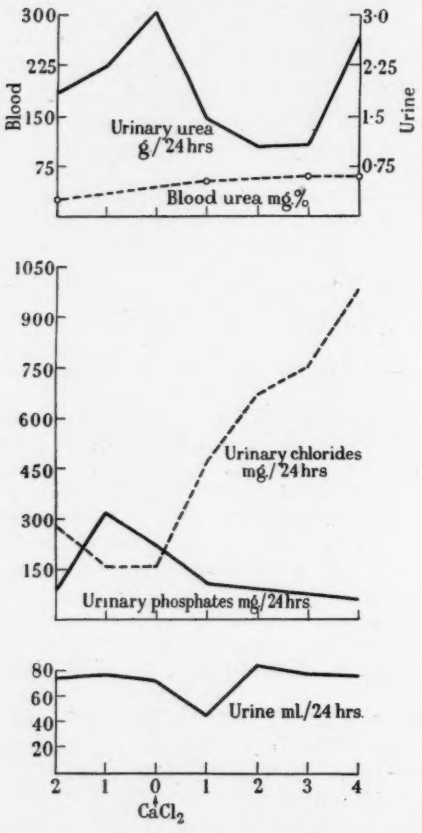


FIG. 1.—Graphs illustrating the effect on Animal 2 of administering 1·5 g. calcium chloride per kg. for 4 days. The animal was killed on the 5th day.

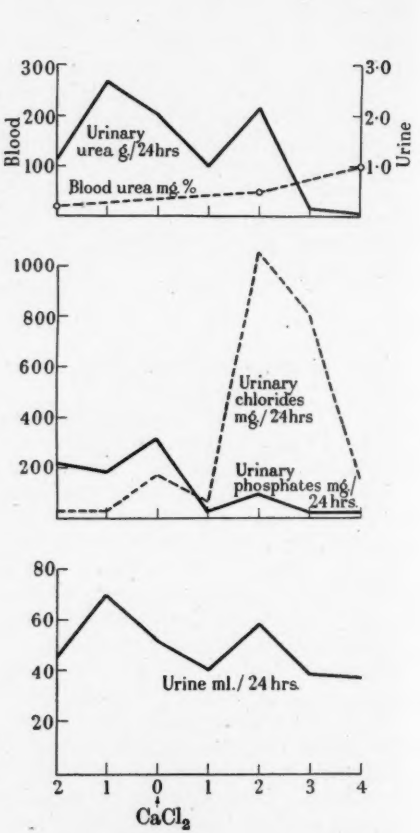


FIG. 2.—Graphs illustrating the effect on Animal 8 of administering 2·5 g. calcium chloride per kg. for 4 days. The animal died on the 5th day.

seen in the heart. The liver was slightly paler than normal, but the spleen showed no abnormality. A distinct zone of pallor was present at the junction of the renal cortex and medulla. Pieces of tissues were taken from all the organs, and fixed and stained as detailed under methods.

Histology.

Lung.—There was marked dilatation of most of the alveoli, but occasional small areas of collapse were present. These areas of collapse were frequently in association with fat emboli, but emboli could also be seen in the emphysematous portions. The number of fat emboli varied in different parts of the lungs, but averaged approximately five per field viewed through a 2/3 in. objective. A marked accumulation of polymorphonuclear leucocytes mainly of eosinphil type was present in the alveolar capillaries, and near the fat emboli these leucocytes appeared to form solid nodules. In one case haemorrhage had occurred into alveoli and bronchi, and free fat globules could be seen in the latter.

Myocardium.—No abnormality could be seen in ordinary paraffin sections of the myocardium. Glycogen was diminished, but most fibres still contained a small amount. Fine fatty globules were present in the myocardial fibres of one animal, but the remainder were normal.

Liver.—No gross abnormality could be seen in paraffin sections, but in some parts the hepatic cells contained collections of greenish-brown granules. Best's carmine stain revealed small deposits of glycogen in the cells surrounding the central veins. Fine droplets of fat were present in the Kupfer cells of the outer and mid zones, and occasionally the parenchymatous cells of the same areas showed a similar change.

Kidney.—The glomeruli seemed to be larger than normal and in many cases slight adhesions appeared to have developed between the tufts and Bowman's capsule. Pronounced catarrhal changes were present in the first convoluted tubules. Distal to this the nephron was dilated and the epithelium of the ascending Henle limb and second convoluted tubule was flattened.

Group 2.

Of 7 animals, 6 received 2·5 g. calcium chloride per kg. and one received 2·2 g. per kg.

Five of these animals died on the 5th day of the experimental period. The remaining two animals, including that receiving 2·2 g. of calcium chloride per kg., were comatose on the same day and were killed. All animals exhibited similar symptoms during the last 24 hours. They became progressively drowsier, gradually losing the power of their limbs and repeatedly falling over. Finally they became comatose and died.

During the first 24 hours of calcium chloride administration urinary volume diminished as in Group 1. Likewise they increased during the second 24-hour period, but by 72 hours they had begun to fall and this fall continued up to the time of death. The excretion of urea ran roughly parallel with that of water, but the rise in urea output at 48 hours did not correspond with the increase in volume. Phosphate showed a progressive fall from its previously high level. Chlorides fell initially, then rose steeply, but during the last 24 hours the output fell once more. The pH, at first reduced, rose, and during the last 2 days was always above 8·0.

In all cases the blood urea rose and the average was around 100 mg. per 100 ml. before death. A marked acidosis was present, and the CO_2 combining power of the blood was reduced to 16 volumes per cent. The pH of the blood dropped, but the fall was no greater than in Group 1. Chlorides increased as before.

Serum phosphorus was doubled and serum calcium also increased. The Ca:P ratio was almost halved. Cholesterol showed a marked increase, almost 200 per cent, and plasma lipoids were augmented by almost 500 per cent. Table II gives a comparison of the average blood values of this group with that of normals.

TABLE II.

	Blood urea, mg./ 100 ml.	CO_2 c.p., vol./ 100 ml.	pH.	Cl', mg./. 100 ml.	Serum Ca., mg./ 100 ml.	Serum P. mg./ 100 ml.	Cholesterol, mg./ 100 ml.	Lipoids, mg./ 100 ml.	Ammonia, mg./ 100 ml.
Normals .	42·1	40	7·19	594·6	12·0	3·76	43·6	0·14	0
After $CaCl_2$.	92	16	7·00	752·6	16·2	8·1	116·0	0·6	5·35

The graph in Fig. 2 shows the changes in Animal 8. At post-mortem the changes were very similar to those seen in Group 1. The depressed, congested areas of collapse in the lung were more numerous but smaller. Marked emphysema was present in the intervening lung tissue. The liver showed a generalized pallor and the lobular markings were blurred. There was well-marked enlargement of the kidneys. The cut surface showed a distinct bulging zone of marked pallor at the junction of cortex with medulla. The cortex also showed pallor, but the medulla was congested. No abnormality could be seen in the other organs.

Histology.

Lung.—Areas of collapse were much more frequent in this group. Fat emboli were present, but were, on the average, only slightly more frequent than in Group 1. There was marked dilatation of the peri-vascular lymphatics, which contained free fat globules. Nodes of eosinophil reaction were numerous (Fig. 3). A few alveolar haemorrhages were present and many of the alveoli contained macrophages, some of which had foamy cytoplasm. In one case a slight fibrinous exudate was present on the pleural surface adjacent to an area showing haemorrhage, oedema and collapse.

Myocardium.—Fine fatty degeneration was present in all cases. Practically no glycogen remained, and the alkaline phosphatase reaction was negative.

Liver.—Glycogen was greatly diminished, but not entirely absent. A few granules could still be seen in the cells surrounding the central vein. Focal degenerative changes were present in the mid-zones of most lobules (Fig. 4).

EXPLANATION OF PLATES.

FIG. 3.—Section of lung from Animal 7, Group 2, showing a solid node of eosinophils in an inter-alveolar septum. H. and E. × 340.

FIG. 4.—Section of liver from Animal 8, Group 2. Note the islands of necrotic cells showing hyperchromatic nuclei. H. and E. × 85.

FIG. 5.—Section of liver from Animal 9, Group 2, showing areas of degeneration with karyolysis of nuclei. H. and E. × 300.

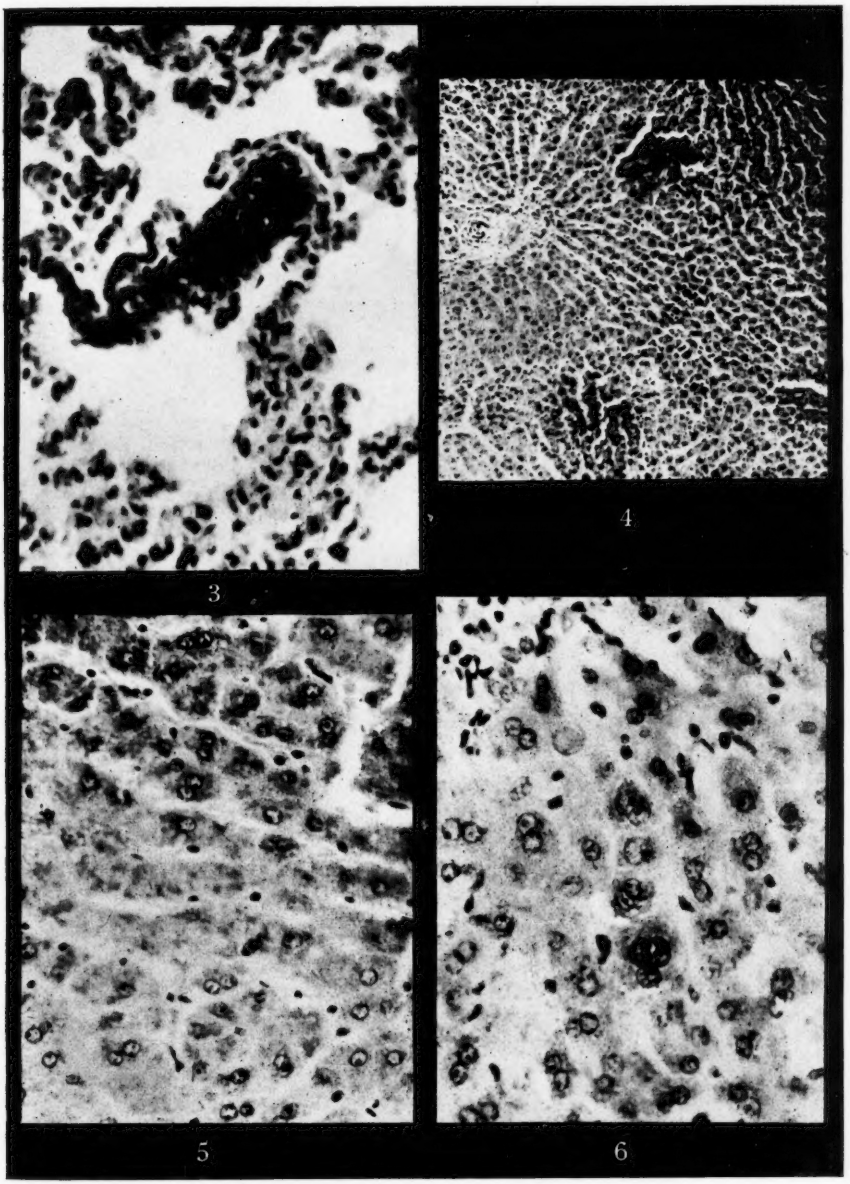
FIG. 6.—Section of liver from Animal 9, Group 2, showing binucleate and trinucleate cells. H. and E. × 340.

FIG. 7.—Section of liver from Animal 11, Group 2, showing greenish-brown granules in cytoplasm of cells. H. and E. × 380.

FIG. 8.—Section of kidney from Animal 9, Group 2, showing degeneration of cells of descending portions of first convoluted tubules. H. and E. × 340.

FIG. 9.—Section of kidney from Animal 8, Group 2, showing dilatation of ascending limbs of Henle. H. and E. × 85.

FIG. 10.—Section of kidney from Animal 10, Group 2, showing pyknosis in the cells of the broad ascending limbs of Henle. H. and E. × 250.



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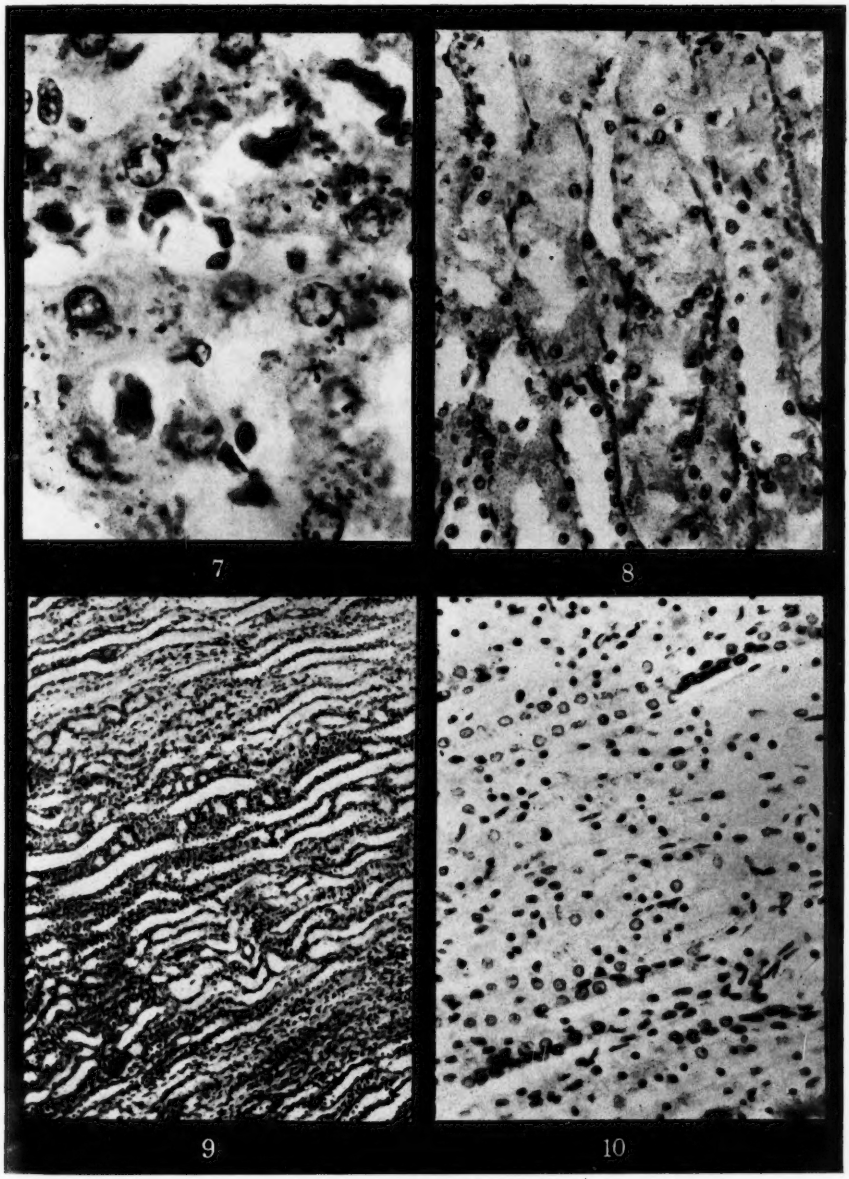
Govan and Parkes.



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Govan and Parkes.

Most commonly these took the form of islands of cells staining strongly with eosin and showing pyknosis of the nuclei. These cells tended to separate from the surrounding columns. Less commonly the nuclei showed karyolysis (Fig. 5), and cytoplasm, staining palely with eosin, was finely vacuolated. Signs of regeneration were present in other parts, the cells being frequently binucleate or even tri-nucleate (Fig. 6). Cells in all parts of the lobules contained greenish-brown granules (Fig. 7). Fat was present in the Kupfer cells. The phosphatase reaction was weak.

Kidney.—The degree of renal damage was very variable in these animals. In all of them the glomerular tufts were enlarged, and in the majority light adhesions had formed between the tufts and Bowman's capsule. Albuminoid material was present in the subcapsular spaces. The capillaries were quite patent, and contained a few eosinophil leucocytes in addition to red cells. Marked catarrh was present in the first convoluted tubules and broad descending limbs of Henle. In some cases the lining epithelium was necrotic, the nuclei showing pyknosis and karyolysis. In a few cells the cytoplasm contained brown granules (Fig. 8). The distal portion of the broad ascending limbs of Henle was greatly dilated (Fig. 9). The intervening portion of the nephrons lying in the juxta-medullary region showed necrosis of many cells, mainly in the first part of the broad ascending limb. Pyknotic changes in the nuclei were marked (Fig. 10). This zone was almost completely bloodless, and corresponded to the zone of pallor seen on the cut surface of the kidneys at post-mortem. Colloid casts were common in the collecting tubules. Some of the collecting tubules showed degeneration of the epithelium.

DISCUSSION.

In our paper on ammonium chloride acidosis (Govan and Parkes, 1946) we stated that at least two theories regarding the cause of death in these animals were possible, namely, that (*a*) the animal died of uraemia as a result of nephritis, or (*b*) they died of ammonia poisoning.

Contrary to the findings in acute ammonium chloride poisoning the renal damage in the present experiment was irregular in extent and occurrence, although the symptoms exhibited and the interval of time between administration of calcium chloride and death of the animal were the same. From this alone it would seem that nephritis is not the primary cause of death in these animals, although no doubt it is a contributory factor.

It has been shown that the blood ammonia appears to be greatly increased after administration of 2·5 g. calcium chloride for 4 days. The possibility of spontaneous formation of ammonia from adenosine phosphate (Conway and Cook, 1939) must be taken into consideration. The original concentration may, in this way, be increased many times in a relatively short interval. Benedict and Nash (1926) and Conway (1935) showed, however, that if precautions were taken to prevent exposure to the air, and the estimations were carried out as soon as possible, then the concentration was usually less than 0·05 mg. per 100 ml. In the present series of experiments all possible precautions were taken to guard against this possibility, and it is significant that no appreciable amount of ammonia could be found in the rabbits' bloods prior to administration of calcium chloride. According to McNeil and Levy (1917–18) the blood ammonia in normal animals is always less than 1 mg. per 100 ml. In the present experiment

it rose almost to 6 mg. per 100 ml. The cause of this increase is not quite clear. It is possible that it represents an attempt to neutralize the acidosis, but in the present series the blood ammonia was only increased in the second group of animals, although the degree of acidosis as determined by the CO_2 combining power of the plasma was no greater than in Group 1. A further proof that the blood ammonia levels bear no relationship to acidosis can be seen in the fact that higher levels were found in animals poisoned with ammonium chloride, although the degree of acidosis was similar to that observed in the present series. These findings are in agreement with those of Markert (1935, 1936).

Alternatively the blood ammonia increase may have been due to a change-over from carbohydrate to fat metabolism, as suggested by the histological appearance in the liver and heart. Many workers, McNeil and Levy among them, have shown, however, that the blood ammonia is not raised in diabetic acidosis, where it is definitely established that fat is being metabolized.

The fact that ammonia appeared in the blood only in the case of animals of Group 2 suggests that its appearance was associated with lesions found in the organs of these animals. In this instance the most marked lesions were those of the liver and kidneys. Ammonia is rapidly converted to urea in the normal animal, and according the Svedberg, Maddock and Drury (1938) the sole source of urea in the rabbit is the liver. There would therefore seem to be strong presumptive evidence in the present experiment that the increased blood ammonia is due to liver dysfunction. Also, McNeil and Levy make a series of observations in their paper which tends to confirm the above. These authors only found a raised blood ammonia in acidosis if the liver was damaged. For instance, no increase of blood ammonia could be found in nephritis unless there was a coincidental myocarditis causing venous congestion of the liver. In 13 cases of acidosis, of which 6 were diabetics, only 2 showed an increased blood ammonia. One was a case of general peritonitis with fatty degeneration of the liver, the other a case of persistent vomiting after laparotomy.

All animals receiving 0·75 g. of ammonium chloride per kg. per day died after 4 days of this treatment. Calculating on the basis of chlorine content the corresponding dose of calcium chloride ought to be 0·76 g. per kg. per day. Instead, the dose required was 2·5 g. per kg. It may be that the chlorine of the calcium salt was not available, but the plasma CO_2-combining power was as low after administration of 1·5 g. of calcium chloride per kg. as it was after 2·5 g. per kg. This would suggest that the ammonium salt is more toxic than the calcium, and this must be by virtue of the ammonium ion.

It seems unlikely that the renal tubular necrosis was directly related to the acidosis, since the changes were minimal in Group 1 and irregular in their extent in Group 2, although all animals were profoundly acidotic. The appearance of the kidneys at post-mortem suggests that the ultimate cause of the tubular necrosis was anoxaemia due to a deviation of the blood from the juxta-medullary region. The cause of this deviation is not apparent, but since the avascular zone was present in both groups, it suggests that the altered blood flow was directly related to the acidosis.

Several minor points arise for discussion. It seems from the histological appearances and biochemical findings that fat is mobilized from the depots long before the glycogen stores are exhausted. Fat was present in the Kupfer cells of the liver and in the myocardium, although both organs contained small

amounts of glycogen. Although the fatty change in the myocardium may be the result of degeneration, recent work (Dible, 1934; Dible and Gerrard, 1938) suggests that the process is always one of infiltration.

It will have been noted that the serum phosphorus and calcium were both raised prior to administration of calcium chloride. In addition the CO_2-combining power of the plasma was reduced. This latter feature was also found in our ammonium chloride series, and it appears to be related to the diet of oats and bran without greens. The former changes are probably related to this mild acidosis. The change in calcium concentration seems to be secondary to that of serum phosphorus. This is suggested by the fact that urinary phosphorus increased during this period. A further significant feature is that serum phosphorus showed a further increase during the period of calcium chloride administration. These changes would appear to indicate that phosphorus plays an important role in combating the acidosis, and that probably phosphorus is mobilized from body stores.

Finally, attention must again be drawn to the occurrence of pulmonary fat emboli in these experiments. The meaning of this is not apparent, but it is important to realize that the reaction of the lungs to emboli is collapse—a fact which may be of some significance in cases of post-operative collapse. It is also interesting to note the intense eosinophil reaction to these emboli.

SUMMARY.

The oral administration of 2·5 g. calcium chloride per kg. daily to rabbits causes death after four days.

This is accompanied by extensive focal necrosis in the liver, and necrosis of tubules in the juxta-medullary region of the kidney.

The results suggest that death may be due to ammonia poisoning, and that the increase in blood ammonia is related to the hepatic lesions.

Following administration of calcium chloride glycogen stores are rapidly depleted, but before this is complete fat is mobilized from the depots.

This mobilization results in histologically demonstrable deposits of fat in the Kupfer cells of the liver and in the myocardium, and it is accompanied by the formation of fat emboli in the lungs.

The fat emboli are accompanied by a marked eosinophil reaction and cause collapse of the pulmonary alveoli.

There is a retention of phosphorus after calcium chloride administration and the calcium-phosphorus ratio is diminished.

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ANTIBIOTICS FROM ASPERGILLI.

DOROTHY GILL-CAREY.

From The Sir William Dunn School of Pathology, Oxford.

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A SUMMARY of the work of a number of investigators on the antibiotics produced by Aspergilli has recently been made (Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949). During the compilation of the tables for this summary it became apparent that some gaps remained to be filled. The object of the present work was to investigate named and identified species which had not, so far, been examined for their capacity to produce antibiotics. The work completes the examination, under certain conditions of growth, of strains of all known species of Aspergilli with the exception of a few so far unobtainable.

Though the examination of fungi for the production of antibiotics is essentially straightforward, it should be emphasized that the statement that a given fungus does or does not produce an antibiotic must be received with a certain reserve, as conditions of growth and testing may greatly affect the result.

Clearly, in a preliminary examination of a number of different cultures it is not practicable to carry out exhaustive experiments on each species. In the following work a certain set of conditions was selected, and each culture tested under approximately the same conditions of growth.

EXPERIMENTAL.

The fungi examined were each grown on all the nine media shown in Table I, which were chosen because they have been found by many observers to allow good growth of many fungi.

TABLE I.—*Media Used.*

1. Potato dextrose . . .	200 g. chopped potato, steamed for one hour with 600 ml. water. Supernatant liquid drawn off and 10 g. glucose added. Made up to 1000 ml. with water.
2. Modified Sabouraud . .	10 g. peptone. 40 g. maltose. 26 g. malt extract. Made up to 1000 ml. with water.
3. Modified glucose Sabouraud .	10 g. peptone. 40 g. glucose. 26 g. malt extract. Made up to 1000 ml. with water.
4. Malt extract . . .	50 g. malt extract. Made up to 1000 ml. with water.
5. Czapek-Dox . . .	3 g. $NaNO_3$. 1 g. KH_2PO_4. 0·5 g. KCl. 0·5 g. $MgSO_4.7H_2O$. ·01 g. $FeSO_4.7H_2O$. 40 g. glucose. Made up to 1000 ml. with water.
6. Czapek-Dox + 5% corn steep	Same as Medium 5, with the addition of 50 ml. corn steep liquor, neutralized and filtered, before making up to 1000 ml.
7. Czapek-Dox + 5% corn steep, neutralized . . .	Same as Medium 6, but brought to pH 7.
8. Beer wort	50 ml. wort, made up to 1000 ml. with water.
(This medium was little used as the beer wort was difficult to obtain.)	
9. Yeast medium . . .	20 g. dried autolysed yeast stirred into 100 ml. warm water. Centrifuged and supernatant liquid poured off. 50 ml. of the supernatant liquid and 40 g. glucose made up to 1000 ml. with water.

Distilled water was used to make up all the above media.

The first experiments were done by growing the fungi at 24° C. in 10 ml. of sterile medium contained in hard glass bottles which were roughly 3 cm. in diameter and 5½ cm. tall. Testing could only be carried out for 12 days, as at the end of that time no medium remained. For investigations involving more prolonged sampling the cultures were planted in 250 ml. Erlenmeyer flasks containing 70 ml. of sterile medium, and were tested for 35 days. Some slow-growing members of the *Aspergillus glaucus* group were grown in hard glass bottles with extra sugar in the media, 20 per cent glucose in Czapek-Dox, potato dextrose, or yeast medium. Such fungi were allowed to grow at room temperature and were tested for up to 50 days.

The metabolism fluid at various stages of growth was tested by the cylinder-plate method (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941 ; Heatley, 1944) with arbitrarily selected bacteria ; strains of *Bact. coli*, *Staph. aureus*, *C. xerosis* and *Myco. phlei* were used. Clearly, production of very specific antibiotics, such as those produced by *Bact. coli* (Heatley and Florey, 1946), may have remained unobserved if an organism sensitive to them was not included.

RESULTS.

The results are set out in Table II.

TABLE II.—*Results.*

Aspergillus.	Strain.	Test organism. *Bact. coli*	*Staph. aureus.*	*Myco. phlei.*	*C. xerosis.*	References from Table I of media on which activity was produced.
***A. GLAUCUS* group.**						
A. repens series.						
A. pseudo-glaucus Bloch	Baarn	−	−	−	−	..
A. ruber series.						
A. proliferans G. Smith	NCTC 6546	−	−	−	−	..
A. chevalieri series.						
A. chevalieri (Mang.) Thom and Church	NRRL 78	−	−	−	−	..
A. chevalieri (Mang.) Thom and Church, var. *intermedius* Thom and Raper	NRRL 82	−	+	+	+	2, 3, 4.
A. amstelodami series.						
A. itaconicus Kinoshita	Baarn	−	−	−	−	..
A. herbariorum series.						
A. carnoyi (Biourge) Thom and Raper	NRRL 126	−	−	−	−	..
A. mangini n. comb.	NRRL 117	−	+	+	+	9 with 20% glucose after 50 days.
A. echinulatus (Delacr.) Thom and Church	Baarn	−	−	−	−	..
A. niveo-glaucus Thom and Raper (syn. *A. glaucus*)	NRRL 127	−	+	−	+	5 with 20% glucose after 35 days.
A. umbrosus Bainier and Sartory	Baarn	−	−	−	−	..
A. restrictus series.						
A. gracilis Bainier	Baarn	−	+	+	+	5 with 20% glucose after 50 days.
***A. NIDULANS* group.**						
A. caespitosus Thom and Raper	NCTC 6972	−	+	+	+	5, 6, 7.
A. nidulans (Eidam) Wint. var. *latus* Thom and Raper	NRRL 200	−	−	−	−	..
A. quadrilineatus Thom and Raper	NRRL 201	−	+	+	+	5, 6, 7.
A. rugulosus Thom and Raper	NRRL 206	−	−	−	−	..
A. unguis (Emile-Weil and Gaudin) Thom and Raper	Baarn NRRL 216	−	+	+	+	5 or 1.
A. variecolor (Berk. and Br.) Thom and Raper	Baarn	−	−	−	−	..

TABLE II (cont.).—*Results.*

Aspergillus.	Strain.	Test organism. *Bact. coli.*	*Staph. aureus.*	*Myco. phlei.*	*C. xerosis.*	References from Table I of media on which activity was produced.
A. USTUS group.						
A. granulosus Raper and Thom	NCTC 6973	−	−	−	−	..
A. VERSICOLOR group.						
A. janus Raper and Thom	NCTC 6970	−	+	+	+	1, 5, 6, 7, 8.
A. janus var. *brevis* Raper and Thom	NCTC 6971	−	+	+	+	1, 5, 6, 7.
A. TERREUS group.						
A. terreus series.						
A. terreus Thom var. *aureus* n. var.	NRRL 1923	−	+	+	+	1 or 4.
A. terreus Thom var. *boedijni* n. var.	NRRL 680	+	+	+	+	1, 2, 3, 4.
A. terreus Thom var. *floccosus* Shih	Baarn	−	+	+	−	4.
A. carneus series.						
A. carneus (v. Tiegh.) Bloch	NRRL 527	−	+	+	+	1, 2, 6.
A. NIGER group.						
A. niger series.						
A. awamori Nakazawa	NCTC 2044	−	−	−	−	..
A. foetidus n. sp.	Baarn	−	−	−	−	..
A. phoenicis (Cda.) Thom	Baarn	−	(+)	(+)	(+)	..
A. carbonarius series.						
A. atropurpureus Zimmerman	Baarn	−	−	−	−	..
A. fonsecaeus n. sp.	NRRL 67	−	(+)	(+)	(+)	..
A. luchuensis series.						
A. japonicus Saito	NCTC 5604*b*	−	−	−	−	..
A. WENTII group.						
A. alliaceus Thom and Church	Baarn	+	+	+	+	4.
A. avenaceus G. Smith	NCTC 6545	−	−	−	−	..
A. panamensis Raper and Thom	NCTC 6974	−	+	+	+	1 or 8.
A. TAMARII group.						
A. terricola series.						
A. lutescens (Bain.) Thom and Church	NRRL 425	+	+	+	+	all.
A. FLAVUS-ORYZAE group.						
A. micro-viride citrinus Cost and Lucet	NRRL 48	−	−	−	−	..
A. OCHRACEUS group.						
A. sulphureus series.						
A. quercinus (Bain.) Thom and Church	NCTC 6979	±	+	+	+	3, 6.
A. sulphureus (Fres.) Thom and Church	Baarn	±	+	+	+	3, 2, 4, 6.
A. ochraceus series.						
A. butyraceae Bainier	Baarn	−	−	−	−	..
A. elegans Gasperini	Baarn	−	−	−	−	..
A. melleus Yukawa	Baarn	±	+	+	+	2, 3, 4, 6
A. sclerotiorum Huber	Baarn	−	−	−	−	..
A. sparsus Raper and Thom	NCTC 6975	−	−	−	−	..

Source of culture ; NCTC = National Collection of Type Cultures, England. Baarn = Centraalbureau voor Schimmelcultures, Baarn, Holland. NRRL = Northern Regional Research Laboratory, Peoria, U.S,A.

Activity ; + active; − inactive; ± very slightly active; (+) activity probably due to low pH as on neutralisation no activity was demonstrable.

The following species of Aspergilli have not been examined, as cultures could not be obtained: (1) *Aspergillus miyakoensis* Nakazawa; (2) *Aspergillus montevidensis* Talice and Mackinnon; (3) *Aspergillus humicola* Chaudhuri and Sachar; (4) *Aspergillus delacroixii* (Sacc.) Thom and Church. Thom and Raper (1945) considered that "it is possible that some old material of a strain of *A. oryzae* might have furnished the type."

SUMMARY.

Thirty-seven species of Aspergilli, comprising 42 strains, which are not known to have been investigated before, have been examined for their ability to produce antibiotics in surface culture on 9 different liquid media. Twenty strains produced metabolism solutions with some antibacterial activity.

I am indebted for the supply of organisms to Dr. K. B. Raper, of the Northern Regional Research Laboratory, Peoria, Professor Dr. Johanna Westerdijk, of the Centraalbureau voor Schimmelcultures, Baarn, and the National Collection of Type Cultures.

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THE NATURE OF SOME ANTIBIOTICS FROM ASPERGILLI.

DOROTHY GILL-CAREY.

From The Sir William Dunn School of Pathology, Oxford.

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DURING a survey of 42 cultures of Aspergilli (Gill-Carey, 1949) several species were discovered which produced antibiotics, and some of these have been investigated further.

General conditions of culture.

All the fungi investigated were grown in stationary cultures in glass vessels on 500 ml. of medium in a layer 2 cm. in depth. Incubation was carried out at 24° C., the activity which developed in the medium being followed by the cylinder-plate method of assay (Heatley, 1944).

Antibiotics from the Aspergillus ochraceus *group.*

Antibiotics were isolated from two closely related species, *A. quercinus* (Bain.) Thom & Church, NCTC 6979, and *A. sulphureus* (Fres.) Thom & Church, Baarn.

A. quercinus.

Medium and conditions of culture.—The fungus was cultivated on glucose-Sabouraud medium containing 1 per cent peptone, 2·6 per cent malt extract and 4 per cent glucose for 15 days.

Isolation.—The active principle was removed from the metabolism solution by shaking with 2 per cent charcoal and eluting with 80 per cent acetone.

The acetone was distilled off under reduced pressure and the remaining aqueous solution shaken with ether at pH 2. On evaporation of the ether, colourless rhombic crystals were obtained and were purified by recrystallization from hot water or petrol ether.

Chemical properties.—The substance recrystallized from petrol ether in the form of colourless needles, m.p. 83° C. On admixture with an authentic sample of penicillic acid, m.p. 83° C., there was no depression in melting point. The substance crystallized from hot water in the form of rhombic plates, m.p. 60° C.

[Birkinshaw, Oxford and Raistrick (1936) report m.p. of 83–84° C. and 64–65° C. for penicillic acid recrystallized from petrol ether and hot water respectively.]

The material was stable at 100° C. in aqueous solution at pH 2, but its antibacterial activity slightly decreased on boiling for 30 minutes at pH 9.

The substance gave no coloration with ferric chloride.

With strong ammonia the substance gave the characteristic pink colour reaction for penicillic acid (Birkinshaw *et al.*, 1936).

The antibacterial properties of the material agreed with the figures given for penicillic acid (Alsberg and Black, 1913 ; Heatley and Philpot, 1947 ; Kavanagh, 1947 ; Oxford, Raistrick, and Smith, 1942).

It was thus evident that this strain of *A. quercinus* produced penicillic acid.

A. sulphureus.

Medium and conditions of culture.—This species was cultivated under similar conditions to *A. quercinus*. The metabolism solution was harvested after 12 days' incubation.

Isolation.—The active principle was extracted in a manner similar to that used for the substance from *A. quercinus*.

Chemical properties.—The substance recrystallized from petrol ether in the form of colourless needles, m.p. 83° C. When mixed with the substance from *A. quercinus* the melting point was 83° C.

With strong ammonia the substance gave the characteristic pink colour reaction for penicillic acid.

It was thus evident that these strains of *A. sulphureus* and *A. quercinus* produced the same substance, penicillic acid.

Antibiotics from the A. wentii *group.*

Antibiotics were isolated from two species in the *A. wentii* group, *A. wentii* Wehmer, IMI 17295, and *A. alliaceus* Thom & Church, Baarn.

A. wentii.

Several strains of *A. wentii* have been tested for antibacterial activity, one by Wilkins and Harris (1942), five by Wilkins (1944), and four by Furtado (1944). Strain 17295 of the Imperial Mycological Institute was tested and found to produce good antibacterial activity when grown on a 5 per cent malt extract broth. Two other strains tested simultaneously, IMI 23010 and IMI 23012, yielded culture fluids with much weaker activity.

Thom and Raper (1945) reported that a strain of *Aspergillus wentii* was found by Karow (1942) to produce citric acid. Yabuta (1912, quoted from Thom and Raper, 1945) reported the production of kojic acid by *A. wentii*, but Birkinshaw, Charles, Lilly and Raistrick (1931) stated that the culture fluid of *A. wentii* gave negative tests for kojic acid. Possibly these divergent results were due to the use of different strains of the organism by different workers.

Medium and conditions of culture.—The fungus was grown on 5 per cent malt extract broth. The metabolism solution was harvested after 15 days' incubation.

Isolation.—The active principle was removed from the culture fluid at pH 6 by continuous extraction with ether. On evaporation of the ether, needle-shaped crystals were obtained. These were recrystallized from aqueous acetone.

Chemical properties.—The substance melted at 150° C. On mixing with an authentic sample of kojic acid (Kemball, Bishop & Co. Ltd.), m.p. 150° C., the melting point was not depressed.

The substance gave a deep red-purple colour with ferric chloride which did not disappear on standing.

The chemical and antibacterial properties of the substance agreed with those given for kojic acid (Birkinshaw, Charles, Lilly and Raistrick, 1931 ; Heatley and Philpot, 1947 ; Jennings and Williams, 1945).

It was thus evident that *A. wentii* IMI 17295 produced kojic acid.

A. alliaceus.

Medium and conditions of culture.—This species was cultivated under similar conditions to *A. wentii*. The metabolism solution was harvested after 10 day's incubation.

Isolation.—The active principle was extracted in a manner similar to that used for the substance from *A. wentii*, IMI 17295. It formed needle-shaped crystals.

Chemical properties.—The substance melted at 150° C. On mixing with an authentic sample of kojic acid (m.p. 150° C.) the melting point was not depressed.

The substance gave a deep red-purple colour with ferric chloride which did not disappear on standing.

The chemical and antibacterial properties of the substance agreed with those given for kojic acid. It was thus evident that this strain of *A. alliaceus* produced kojic acid.

Antibiotics from A. nidulans *group.*

Penicillin production was demonstrated in two species in the *A. nidulans* group, *A. caespitosus* Thom and Raper, NCTC 6972, and *A. quadrilineatus* Thom and Raper, NRRL 200. A second antibiotic was produced by *A. caespitosus.*

A. caespitosus.

Medium and conditions of culture.—The fungus was grown on modified Czapek-Dox medium containing 0·3 per cent $NaNO_3$, 0·1 per cent KH_2PO_4, 0·05 per cent KCl, 0·05 per cent $MgSO_4.7H_2O$, 0·001 per cent $FeSO_4.7H_2O$ and 4 per cent glucose, made up with tap water.

After 10 days the metabolism solution produced a zone of inhibition of about 20 mm. diameter on plates seeded with the H strain of *Staph. aureus.* This activity was destroyed completely by the addition of a few drops of penicillinase to the test solution. After 20 days, zones of about 15 mm. were obtained, and this activity was not destroyed by the addition of penicillinase. Zones of inhibition were just detectable after incubation for 25 days.

A slight purple colour developed in the medium about the ninth day, and after 15 days it was deep purple.

Some culture fluid was harvested after 10 days' and some after 20 days' incubation.

The antibiotic present after 10 days' incubation.—The active principle was unstable. Complete destruction occurred on heating to 100° C. for 15 minutes at pH 2 or 9. Some destruction occurred after 30 minutes at 20° C.

The active principle was extracted into ether at pH 2 (at 5° C.) and back into phosphate buffer at pH 7.

The activity was destroyed completely on incubation with penicillinase solution or copper sulphate solution (Abraham and Chain, 1942).

The antibiotic present after 20 days' incubation.—The active principle was relatively stable. Destruction occurred on heating to 100° C. for 15 minutes at pH 2 but not at pH 9.

It was precipitated with HCl at pH 2 in association with a purple pigment.

Thus a penicillin-like substance was produced by this strain of *A. caespitosus* after 10 days, and was followed by another antibiotic after 20 days' incubation.

A. quadrilineatus.

Medium and conditions of culture.—This species was cultivated under similar conditions to *A. caespitosus*. The metabolism solution was harvested after 15 days' incubation.

Nature of antibiotic produced.—The active principle was unstable. Complete destruction occurred on heating to 100° C. for 15 minutes at pH 2 or 3. Some destruction occurred after 30 minutes at 20° C.

The active principle was extracted into ether at pH 2 (at 5° C.) and back into phosphate buffer at pH 7.

The activity was destroyed completely on incubation with penicillinase solution or copper sulphate solution (Abraham and Chain, 1942).

Thus a penicillin-like substance is produced by this strain of *A. quadrilineatus*.

SUMMARY.

The antibiotics produced by one strain each of six species of Aspergilli were investigated. Two species from the *A. ochraceus* group, *A. quercinus* (Bain.) Thom and Church, and *A. sulphureus* (Fres.) Thom and Church, were found to produce penicillic acid; two species from the *A. wentii* group, *A. wentii* Wehmer (strain IMI 17295), and *A. alliaceus* Thom and Church, were found to produce kojic acid, and two species from the *A. nidulans* group, *A. caespitosus* Thom and Raper and *A. quadrilineatus* Thom and Raper, were found to produce penicillin-like substances. *A. caespitosus* produced also another antibiotic.

I am grateful to Professor H. W. Florey, Dr. N. G. Heatley, Dr. R. Q. Marston, Mr. H. S. Burton, and Mr. G. G. F. Newton for advice on this work, and to Miss Jessie Davidson for technical help.

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THE EFFECT OF TRIPHENYLMETHANE DYES ON THE INTRACELLULAR GROWTH OF INFLUENZA VIRUS A.*

L. HOYLE.
From the Public Health Laboratory, Northampton.

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IN a recent study of the growth cycle of influenza virus A in the fertile egg (Hoyle, 1948) it was shown that growth of virus occurred in a series of intracellular cycles, each cycle lasting about 6 hours. Infective red cell agglutinating virus introduced into the allantoic sac was rapidly taken up by the cells of the chorio-allantoic membrane, but appeared to be converted into a non-infective and non red cell agglutinating form. This intracellular form of the virus then multiplied for a period of 6 hours, at the end of which time a much larger amount of infective virus appeared in the membrane and was almost immediately excreted into the allantoic fluid. Although virus could not be demonstrated in the chorio-allantoic membrane by agglutination tests during the period of intracellular multiplication, nevertheless the virus multiplication could be followed by reason of the appearance and rapid increase in amount of complement-fixing soluble antigen in the membrane during this period.

It was suggested that the influenza virus existed in two forms, an extracellular infective form which agglutinated red cells, and an intracellular multiplying form which did not agglutinate red cells and was possibly identical with the soluble antigen. Whether this theory was correct or not, it seemed evident that the production of soluble antigen in the chorio-allantoic membrane was related to the intracellular multiplication of virus and could be used as a measure of that multiplication. If eggs were inoculated to the allantoic sac with a large dose of

* A Report to the Medical Research Council.

virus sufficient to infect simultaneously all the available cells, then the production of soluble antigen in the chorio-allantoic membrane was remarkably constant, both in rate of production and total amount produced. It seemed that there was here available a very convenient technique by means of which the effect of any substance on the intracellular growth of the influenza virus could be studied. This paper describes the results of such experiments with a variety of dyes, in particular with dyes of the triphenylmethane group.

Technical Methods.

A highly egg-adapted strain of virus (D.S.P.) was used throughout the work. Twelve-day-old fertile eggs were inoculated to the allantoic sac with 0·3 ml. of undiluted allantoic fluid from an egg inoculated 18 hours previously with D.S.P. virus. This very large inoculum was more than sufficient to infect all the available cells. The eggs were incubated 30 minutes to allow infection to take place, and then the dye to be tested was inoculated to the allantoic fluid. The eggs were then incubated for a further 5½ hours, and the chorio-allantoic membranes removed, washed in saline to remove allantoic fluid, suspended in 1 ml. of saline containing 0·08 per cent sodium azide, and frozen and thawed three times to liberate the soluble antigen. The fluids were then centrifuged and the supernatants tested for soluble antigen content by complement fixation with human convalescent serum. It was found to be unnecessary to remove infective virus from the fluids by red cell adsorption before carrying out the complement-fixation tests, since in these experiments with very heavy inocula the amount of infective virus produced in 6 hours was too small to give appreciable complement-fixation, practically the whole of the complement-fixing power of the extracts being due to soluble antigen.

In each experiment control eggs were tested in which no dye was added. In 30 such control eggs the average titre of complement-fixing antigen in the membrane extract at 6 hours was 1:36 with extremes of 1:14 and 1:80. It was therefore considered that in the experiments with added dyes a titre of 1:10 or less could be taken as indicating a significant reduction of intracellular growth. The titre was taken as that concentration of antigen giving 50 per cent fixation and was determined by the methods described by Hoyle (1945).

Preliminary Experiments.

Attention was first directed to the possible effect on intracellular growth of virus of dyes commonly used in vital staining methods. A range of such dyes was tested in doses of 1 mg. The dosage corresponded to a concentration of dye in the allantoic fluid of about 1:10,000. It was found that at this concentration all the acid dyes tested were non-toxic to the embryo and none produced any effect on the production of soluble antigen. Basic dyes proved to be more toxic, and in some cases the test had to be repeated with smaller doses of dye. With dahlia violet it was found that the production of soluble antigen was markedly reduced even by doses of only one-tenth of the toxic dose. This observation led to a special study being made of the basic dyes of the triphenylmethane group.

TABLE I.—*Titration of Complement-fixing Antigen in Chorio-allantoic Membrane Extracts of Eggs Infected 6 Hours Previously with a Large Dose of Influenza Virus A, the Eggs being Treated 30 Minutes after Inoculation with Varying Amounts of Dahlia Violet.*

Egg.	Amount of dahlia violet (mg.).	State of embryo at 6 hours.	Antigen control.	Haemolysis with antigen dilutions of—							Antigen titre.
				1:1	1:2	1:4	1:8	1:16	1:32	1:64	
1	0	Alive	++++	−	−	−	−	−	−	++++	48
2	0·5	Dead	++++	+++	++++	++++	++++	++++	++++	++++	nil
3	0·25	Alive	++++	++	++++	++++	++++	++++	++++	++++	1
4	0·125	„	++++	−	−	−	++++	++++	++++	++++	6
5	0·06	„	++++	−	−	++	++++	++++	++++	++++	4
6	0·03	„	++++	−	−	−	++++	++++	++++	++++	6
7	0·015	„	++++	−	−	−	−	+	++++	++++	20
8	0·0075	„	++++	−	−	−	−	−	+++	++++	28

Serum: Human convalescent titre 1 : 16; used 1 : 3; control ++++.
++++ = Complete haemolysis, +++, ++, + = intermediate degrees of haemolysis; − = no haemolysis.

The following is a list of dyes which were found to be without effect on soluble antigen production in doses of 1 mg.:

Acid fuchsin.	Neutral red.
Brilliant cresyl blue.	Phenolphthalein
Chlorazol black.	Phenolsulphonephthalein.
Isamine blue.	Rosolic acid.
Janus green.	Trypan blue.
Light green.	Vital new red.
Methylene blue.	

Reduction of Soluble Antigen Production in the Chorio-Allantoic Membrane by Basic Dyes of the Triphenylmethane Group.

Table I shows the results of titrating the complement-fixing antigen in chorio-allantoic membrane extracts from infected eggs treated with varying amounts of dahlia violet 30 minutes after inoculation. The dye proved to be toxic to the embryo in doses of 0·5 mg., but considerable suppression of soluble antigen production occurred even in doses of 0·06 or 0·03 mg.

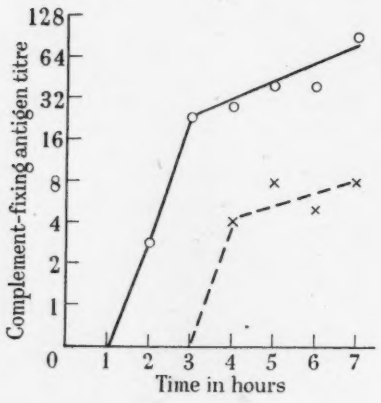

FIG. 1.

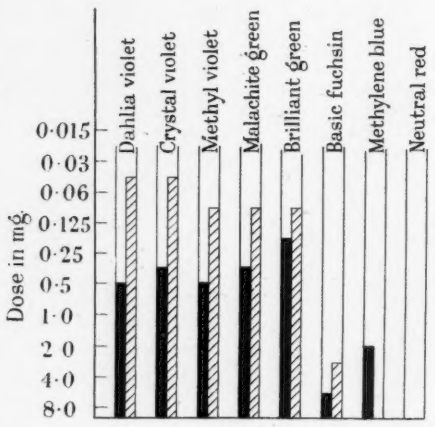

FIG. 2.

FIG. 1.—Effect of 0·1 mg. of dahlia violet added 30 min. after inoculation on rate of production of soluble antigen in chorio-allantoic membranes of eggs inoculated with a large dose of influenza virus A.

Control eggs without addition of dye o———o.
Eggs treated with dahlia violet x - - - x.

FIG. 2.—Effect of basic dyes on production of soluble antigen in chorio-allantoic membrane. Doses toxic to embryo in 6 hours indicated in solid black. Doses producing significant reduction of soluble antigen production indicated by shading.

Fig. 1 shows the effect of 0·1 mg. of dahlia violet on soluble antigen production at various intervals of time. A batch of eggs was inoculated with 0·3 ml. D.S.P. virus allantoic fluid, and 30 minutes later 0·1 mg. of dahlia violet was added to half the eggs. Eggs were then sampled at hourly intervals, and the soluble antigen

content of the chorio-allantoic membranes measured. The production of soluble antigen was reduced and retarded in the eggs treated with dahlia violet.

Similar experiments were done with other basic triphenylmethane dyes and the results are shown in Fig. 2, with, for comparison, results obtained with methylene blue and neutral red. All six of the basic triphenylmethane dyes produced significant reduction in soluble antigen production in doses which were non-toxic to the embryo, but the dyes were not equally effective. The violet dyes gave the best results, the green dyes were more toxic and less effective, while basic fuchsin was much less toxic than the other dyes and was only very slightly effective. Methylene blue had no effect on soluble antigen production even in doses which killed the embryo in 6 hours. With this dye the embryo usually survived for some hours even after the addition of toxic doses, and in this time production of soluble antigen occurred. Neutral red was non-toxic even in doses of 8·0 mg. and had no effect on soluble antigen production.

Nature of the Effect of Basic Triphenylmethane Dyes on Soluble Antigen Production.

The experiments described show that in suitable doses certain basic dyes of the triphenylmethane group will greatly reduce the production of soluble antigen in the chorio-allantoic membrane of eggs inoculated with influenza virus A. This effect might be produced in three ways:

(1) The dye might prevent infection of the cells.

(2) The dye might destroy the soluble antigen.

(3) The dye might interfere with the metabolism of the infected cell in such a way as to reduce the intracellular multiplication of the virus.

In all the experiments the eggs were inoculated with a very large dose of virus, and a period of 30 minutes allowed before addition of the dye. Under these circumstances it seems impossible that the dye should have been able to prevent infection of the cells. Also the red cell agglutinating power of virus is unaffected by concentrations of dye which are effective in suppressing soluble antigen production in the infected egg. Thus a concentration of crystal violet of 1:100,000 in the allantoic fluid markedly reduced soluble antigen production, but it required at least ten times this amount to produce any effect on the ability of virus to agglutinate red cells. The infectivity of the virus was also unaffected by 30 minutes' exposure to 1:100,000 crystal violet; a 1:20 dilution of D.S.P. virus allantoic fluid so treated was still infective in a dilution of 1:100 million. The infectivity was considerably reduced by 1:10,000 crystal violet and was destroyed by 1:1000. The soluble antigen is even more resistant than the agglutinin *in vitro*; thus with crystal violet it required a concentration of 1:2000 to reduce the complement-fixing power of a chorio-allantoic membrane preparation of soluble antigen by 50 per cent. It seems probable, therefore, that the dyes do not act directly either on the infective virus or on the soluble antigen, but that the effect is due to an interference with the metabolism of the infected cell. It is probable that these dyes do have a pronounced effect on cell metabolism, since they are more toxic to the embryo than the other dyes tested.

Effect of Basic Triphenylmethane Dyes on Production of Infective Virus in Eggs.

Since the basic triphenylmethane dyes appear to retard and reduce the intracellular multiplication of virus, it might be expected that the liberation of extracellular infective virus into the allantoic fluid would also be reduced. Eggs were inoculated with 0·1 ml. of a 1:10 dilution of D.S.P. virus allantoic fluid, and 30 minutes later 0·1 mg. of dye was added. The allantoic fluid was sampled at intervals and the red cell agglutinin titre measured by the Salk test, and compared with control eggs without dye. The results are shown in Fig. 3. The liberation of virus into the allantoic fluid was retarded in the eggs treated with dye, though the final titre attained was not greatly reduced. It was noted in these experiments that the dyes were fairly rapidly destroyed in the egg. Thus, with crystal violet,

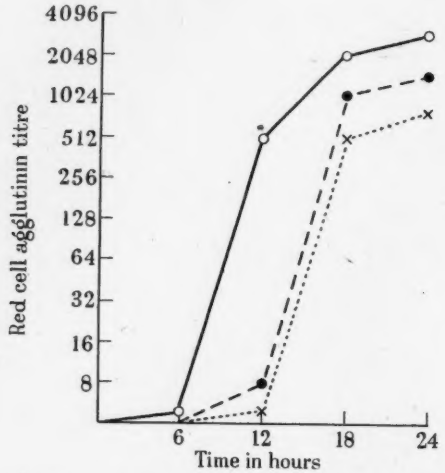

FIG. 3.—Effect of 0·1 mg. of crystal violet and 0·1 mg. of malachite green added 30 minutes after inoculation on the rate of liberation of virus into the allantoic fluid in eggs inoculated with a small dose of influenza virus A.

Control eggs without dye o———o.
Eggs treated with malachite green ● - - - - ●
Eggs treated with crystal violet x . . . . x.

the chorio-allantoic membrane was deeply stained at 6 hours, but by 24 hours all staining had disappeared. It is probable that the intracellular multiplication of virus is delayed until the concentration of dye is reduced below the effective level, and that after this, multiplication proceeds normally so that the final titre of virus in the allantoic fluid is only a little less than in the controls.

DISCUSSION.

The experiments described in this paper show that basic dyes of the triphenylmethane group, introduced in suitable doses into the allantoic sac of eggs infected previously with influenza virus A, are able to retard and reduce the intracellular

growth of the virus, as shown by a reduction in the production of soluble antigen in the chorio-allantoic membrane, and a retardation in the liberation of infective virus into the allantoic fluid. The effect appears to be restricted to the basic dyes of the triphenylmethane group, the violet dyes being most effective. These dyes, however, are more toxic to the embryo than other dyes, and as the effect is only shown by doses of about one-eighth to one-tenth of the toxic dose, it is unlikely that the dyes would have any therapeutic value in influenza. In fact, Andrewes, King and Van den Ende (1943) have already found them to be without chemotherapeutic value in mice infected with influenza virus.

The basic triphenylmethane dyes differ from other dyes in their ability to combine with ribonucleic acid. It is to this property that they owe their efficacy in Gram's method of staining bacteria (Henry and Stacey, 1943). Knight (1947) has shown that influenza virus A contains ribonucleic acid. If ribonucleic acid is an essential constituent of the influenza virus, then it would seem possible that the effect of basic triphenylmethane dyes in retarding the intracellular growth of influenza virus A might be due to an interference with the metabolism of ribonucleic acid in the infected cell.

SUMMARY.

A method is described whereby the effect of any substance on the intracellular growth of influenza virus A can be readily observed by measuring the effect of the substance on the production of complement-fixing soluble antigen in the chorio-allantoic membrane of eggs inoculated with a large dose of virus.

It is shown that basic dyes of the triphenylmethane group, especially dahlia violet and crystal violet, can, in suitable doses, greatly retard and reduce the intracellular growth of the virus. These dyes are, however, more toxic than other dyes to the embryo, and it is unlikely, therefore, that they would be of any chemotherapeutic value in influenza.

It is suggested that the effect of these dyes may be due to an interference with the metabolism of ribonucleic acid in the infected cell.

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AN ANTIBIOTIC PRODUCED BY *STAPHYLOCOCCUS AUREUS*.

JOAN F. GARDNER.

From The Sir William Dunn School of Pathology, Oxford.

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MANY reports have appeared during the last sixty years of the inhibitory or lytic power of staphylococci. In some cases it is clear that an antibacterial substance was concerned, but its properties have varied widely, being sometimes thermostable and sometimes very easily destroyed by temperatures below 100° C.; sometimes soluble and sometimes insoluble in alcohol and other organic solvents; sometimes produced only on solid or on liquid medium and sometimes equally well on either. As this literature has been reviewed elsewhere (Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949) it is not proposed to consider it further here.

Jennings and Sharp (1947) of this laboratory examined several strains of staphylococcus for antibacterial activity, using the streak-plate test with *C. xerosis*, *C. diphtheriae*, *Bact. coli* and *Staph. aureus* NCTC No. 6571 as test organisms. Twenty-six of the 205 strains so examined were found to be active and from one of them, No. E 755, an attempt was made to extract the antibiotic. The results of this work were summarized by Florey *et al.* (1949, Chap. 11) as follows:

> "It was found . . . that an abundant supply of oxygen was necessary for the production of an antibiotic in liquid culture." This had been reported independently by Magrassi and Spiga, 1946. "It was not, however, possible to produce it by bubbling either air or oxygen through liquid cultures, for it was subsequently discovered that the act of bubbling through a gas, even nitrogen, inactivated a solution of the inhibitor. The difficulty was overcome by maintaining an atmosphere rich in oxygen over the surface of the culture.
>
> "It was found that production was most satisfactory in Lemco broth, and was slightly improved by the addition of 1 per cent lactic acid (neutralized). Activity was judged by the inhibitory action against *C. xerosis*. The active substance was not destroyed by boiling for 5 minutes at pH 8. It could be precipitated by addition of an equal volume of saturated ammonium sulphate, though with some loss in activity. Treatment of the dried residue from an aqueous solution with 80 per cent alcohol, followed by evaporation of the alcohol and resolution in water, caused complete inactivation. At pH 4·5 a considerable amount of protein material was precipitated from the medium while all activity remained in solution. Adsorption on to alumina (both untreated and acid-washed) occurred, but attempts to elute with water were unsuccessful. The substance was not dialysable and could be concentrated satisfactorily from Lemco broth or gelatin digest medium by pressure filtration through cellophan."

The present paper reports the results of further work on this problem.

EXPERIMENTAL.

Production of the Antibiotic in Liquid Medium.

Media.

Lemco broth and casein hydrolysate medium were both satisfactory with regard to growth of the organism and antibiotic production. The level of activity reached was slightly higher in Lemco broth, but the high protein content of this medium was a disadvantage, as far as purification of the antibiotic was concerned, in view of the probable protein nature of the latter. However, it was used in preliminary experiments to determine the properties of the antibiotic and the best method of culture of the organism.

Lemco broth.—This contained Lab. Lemco 1 per cent, Eupepton 1 per cent and sodium chloride 0·5 per cent, made up in tap water, the pH being 7·6. In early experiments lactic acid (1 per cent, neutralized) was included, but it was later ascertained that its presence did not enhance production and it was subsequently omitted.

Casein digest medium.—The use of casein hydrolysate as a source of amino acids provided a medium initially free from material which was precipitable by the reagents used in concentrating the antibiotic. A tryptic digest was prepared as follows :

Light, white, soluble casein (180 g.) was dissolved with the aid of a mechanical stirrer in 3 l. of tap water containing 10 ml. of 40 per cent caustic soda solution. The pH was adjusted approximately to 9·0 and 15 g. of commercial trypsin were stirred in. The mixture was transferred to stoppered flasks, which were placed in a water bath at 52° C., toluene being added as a preservative. Digestion was generally allowed to proceed for 24 hours at this temperature, although a 6-hour digest (to which no toluene was added) appeared to be equally satisfactory. Alkali was added at intervals during the first few hours of the digestion period to maintain the pH between 8·0 and 9·0. In the later stages there was no further tendency to shift to the acid side. Small amounts (0·5–1·0 g.) of trypsin were added from time to time to replace any that became inactivated at the high temperature of the mixture.

At the end of the digestion period the mixture was adjusted to pH 6·5 and after removal of the precipitate of undigested casein it was boiled until the volume had been reduced to half. This inactivated the trypsin and eliminated the toluene. The resulting mixture was filtered hot through a coarse filter paper and was made up to 6 l. (≡ 3 per cent casein).

Addition of the following salts completed the medium.

K_2HPO_4	27·0 g.
KH_2PO_4	2·7 g.
$FeSO_4.7H_2O$	0·3 g.

As initial alkalinity of the medium tended to result in a better yield of the antibiotic, it was adjusted to pH 7·5 before autoclaving and the copious flocculant precipitate removed by filtering through a coarse filter paper. The inclusion of glucose (0·25 per cent) in the medium before autoclaving caused an intense brown colour to develop, so this ingredient was autoclaved separately as a 25 per cent solution which was added afterwards with sterile precautions.

Method of culture.

The most effective method of production, and the one which was adopted as a routine procedure, was to grow the organism in shallow layers of medium in vessels equipped to permit continuous or intermittent passage of oxygen over the surface of the medium, but not allowing bubbling through the medium itself.

The organisms were grown in the rectangular porcelain vessels originally used for the production of penicillin (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941). The spout of each vessel was fitted with a rubber stopper pierced by glass inlet and outlet tubes and a third tube to which was connected a glass bulb plugged with cotton wool. This was used to hold the

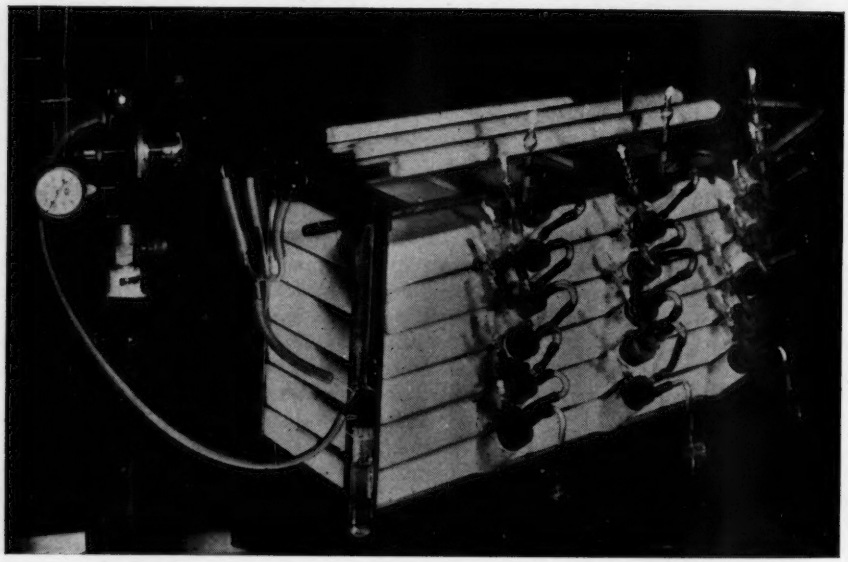

FIG. 1.—Apparatus used for the culture of *Staph. aureus* No. E 755 in shallow layers of liquid medium under an atmosphere rich in oxygen.

concentrated glucose solution which was allowed to run into the vessel after autoclaving by releasing a screw clip on the rubber connection. The inoculum was introduced by the same route. Each vessel contained 300 ml. of medium, which formed a layer of 0·5 cm. deep, this being the maximum depth which consistently resulted in good production (Fig. 1).

A metal frame was designed to hold 18 vessels in 3 piles of 6. The vessels of each pile were connected in series so that oxygen entered the topmost one and passed through each in turn. The bottom vessel of each pile was fitted with a glass tap by means of which the oxygen outlet tube could be closed. Each pile of vessels had its own cotton-wool filter 30 inches long, the three filters being connected by means of a manifold to a single wash bottle and thence to the

oxygen cylinder. The apparatus was completely assembled before autoclaving except that the connection between the wash bottle and the manifold was left open to ensure efficient sterilization of the filters. The taps on the oxygen outlet tubes were also left open, cotton-wool plugs being inserted to prevent contamination. It proved necessary to wrap the necks of the vessels with lint and cotton-wool so as to cover the rubber stoppers, and also the outlet tubes and taps, in order to prevent occasional contamination during the culture period.

After inoculation the apparatus was incubated at 37° C. for 5 days, oxygen being passed through at a rapid rate for five minutes once each day. Increasing the frequency of this procedure did not enhance production.

Method of assay.

The cylinder-plate method (Abraham *et al.*, 1941) was used, Lemco agar plates being surface-seeded with *C. xerosis*. The zones of inhibition were characterized by clearly-defined edges and no resistant colonies appeared. It was not feasible to set up a standard of this antibiotic, but sufficiently accurate results were obtained by measuring the zone diameters from two-fold dilutions of the solutions to be assayed, with particular attention to the highest dilution at which any inhibition was observed. The curve relating zone diameter to dilution was rather flat.

At the end of five days' incubation the culture fluid generally produced a zone of inhibition of 16 mm. diameter and gave a small but measurable zone when diluted 1 in 8.

Extraction and Concentration of the Antibiotic.

Addition of concentrated hydrochloric acid to either a Lemco or casein hydrolysate culture until its pH had fallen to 3·0 caused a copious precipitate of inactive material to form. This was removed, together with the bacteria, by centrifuging and the clear supernatant, when neutralized, contained all the activity.

The procedure for concentration of the antibiotic from cultures which had been clarified in this way varied according to the medium in which the organism had grown.

Concentration from Lemco medium.

Tenfold concentration was brought about with negligible loss of activity by evaporation from a shallow tray in a current of air at 56° C. in a Faust-Heim apparatus. The concentrated solution, after being freed from solid material deposited during evaporation, yielded a copious precipitate when adjusted to pH 3 with concentrated hydrochloric acid. Excess acid caused it to redissolve. If the solution was at least ten times as concentrated as the original culture fluid, this precipitate contained all the activity. It redissolved in water at pH 7 to give a clear, dark brown solution which gave larger and more clearly defined zones of inhibition than did the concentrated solution before acidification, possibly because of less interference with diffusion. Addition of an equal volume of 10 per cent trichloracetic acid to this solution of the antibiotic precipitated the latter with relatively little loss.

Concentration from casein hydrolysate medium.

When formed in this medium the antibiotic was not stable to evaporation at 56° C., and trichloracetic acid was therefore added to the neutral culture fluid, which had been clarified by centrifuging at pH 3, as described above. The details of the method were as follows :

Sufficient trichloracetic acid was added (dissolved in a small volume of water) to give a final concentration of 6 per cent. The slightly cloudy mixture was allowed to stand 15 to 30 minutes and was then centrifuged. The small precipitate could be dissolved in one-sixtieth of the original volume of distilled water by bringing the pH to 7·0. It usually contained from 25 to 50 per cent of the original activity—the higher the activity of the culture fluid, the better was the relative yield on precipitation.

The redissolved precipitate was dialysed against running water for at least 6 hours (dialysis could be continued for 18 hours without noticeable loss occurring), a process which halved the total solids without loss of activity. A further small amount of inactive material was eliminated by placing the dialysed solution (contained in thin-walled test tubes) in a boiling water bath for 3–5 minutes, cooling rapidly and removing the inactive precipitate by centrifuging.

The solution was then dried from the frozen state, yielding about 0·1 g. of voluminous, almost white, non-hygroscopic material per litre of original culture fluid.

Addition of an equal volume of a saturated solution of ammonium sulphate to a cooled neutral solution of the active substance (10 mg./ml.) yielded a precipitate which could be redissolved, dialysed and dried from the frozen state. It contained twice as much active substance per unit weight as did the material precipitated by trichloracetic acid, but the procedure involved disappearance of half the total activity. A more bulky precipitate resulted when the precipitation was carried out at pH 3, but the total yield was no higher.

Stability and Properties of the Antibiotic.

Variations in temperature and hydrogen ion concentration.

The active substance was characterized by considerable stability towards heating at neutral or acid pH values, but was rapidly destroyed if heated in alkaline solution. Cultures could be rendered sterile by holding at 70° C. for 20 minutes without loss of activity. Dilute and concentrated solutions derived from casein hydrolysate cultures were unaffected by heating in a boiling water bath for periods up to 1 hour at pH 3·0, or for at least 10 minutes at pH 7·0. Some activity remained even after autoclaving at 120° C. for 20 minutes at pH 3·0. In general the stability seemed to be slightly greater at pH 3 than at pH 7.

Between pH 7 and pH 9 no decrease of activity occurred during one hour at room temperature, but on heating in a boiling water bath the loss was rapid and complete. Above pH 9 loss of activity was noticeable at room temperature, hence, when neutralizing solutions, care had to be exercised in order to avoid adding excess alkali.

Action of proteolytic enzymes.

The action of commercial and crystalline trypsin at 37° C., pH 8 and in concentration of 1 mg. per ml. resulted invariably in total inactivation within five

minutes of solutions containing up to 10 mg. of dried preparations of the antibiotic per ml. The action of 1 per cent pepsin at pH 3 was not so rapid, as up to 25 per cent of the original activity remained after 30 minutes at 37° C., and traces still remained after 1 hour.

Bubbling and mechanical agitation.

As has already been reported by Florey *et al.* (1949), bubbling nitrogen or air through an active solution under sterile conditions brought about its inactivation. The destruction was a gradual process being complete in 3 hours in the case of a culture in either Lemco or casein digest medium. The same result was obtained at pH 3 and also in the presence of protective agents, such as glycine or gelatin.

Mechanical shaking did not appear to exert such a marked destructive affect as did bubbling, and culture fluids proved to be unaffected by the brief though intense foaming that occurred during passage through a Sharples centrifuge at an acid pH value.

Concentrated solutions of the antibiotic were less rapidly inactivated by bubbling and shaking than were the crude culture fluids, as might be expected if the inactivation were due to a surface denaturation.

Drying.

A cell-free Lemco broth culture withstood evaporation to dryness in a current of air at 56° C., though with casein hydrolysate cultures inactivation occurred under these conditions. Concentrated solutions of active material which had been precipitated in various ways could be dried from the frozen state without loss, but this was not the case with dilute solutions. The dried material could be stored in the refrigerator for several weeks without loss of activity taking place.

Dialysis.

Concentrated solutions could be dialysed in cellophan bags at room temperature for at least 15 hours without loss. Dilute solutions sometimes underwent loss under these conditions, but this was possibly due to the intervention of other agencies, such as contaminating bacteria.

Filtration.

Solutions could be passed through a sintered glass bacterial filter with little loss of activity, but gradocol membranes, like asbestos filter pads, removed the greater part of the active substance.

Ammonium sulphate.

Addition of ammonium sulphate either in solid form or as a saturated solution, and under various conditions of concentration, temperature and pH to a clarified culture fluid resulted in complete loss of activity from both the precipitate and the remaining liquid. A concentrate from Lemco broth behaved in a similar manner, though in the presence of high glycine concentrations (up to 2 M) half the activity could be demonstrated in the precipitate. The remainder had disappeared. Treatment of a concentrated solution of the dried material, obtained by precipitation with trichloracetic acid from casein hydrolysate cultures, with ammonium sulphate in the absence of glycine gave a 50 per cent yield in the precipitate.

Organic solvents.

The antibiotic was characterized by marked instability towards organic solvents, especially in dilute solution. As the degree of purification increased greater apparent stability in the presence of certain solvents was noted.

Culture fluids, or dried residues from them, could not be treated with alcohol at 4° C. without total loss of activity taking place. The material precipitated by trichloracetic acid was inactivated less rapidly in the presence of alcohol in concentrations up to 70 per cent, though the extent of the loss was variable and 80 per cent alcohol caused total inactivation. The antibiotic was soluble in 70 per cent ethyl alcohol, but, owing to its destruction, it was not possible to determine whether or not it was soluble in absolute alcohol and in various other solvents. Methyl alcohol, butyl alcohol and acetone exerted a similar deleterious effect.

If a solution were shaken gently for a few minutes with an equal volume of ether, little activity could be detected in either the aqueous or ether fraction though, as was the case with alcohol, there was evidence of apparently increasing stability of the antibiotic, as more concentrated solutions became available.

Chloroform exerted the most marked destructive effect of all the solvents tested, irrespective of the degree of concentration and purity of the solution. Introduction of a few drops, without shaking, brought about loss of half the activity almost immediately and inactivation was complete within 7 hours.

The known properties of the antibiotic suggest that it is an unstable protein of relatively high molecular weight. The impure state of the preparations used makes it impossible to draw any more definite conclusion.

Antibacterial Activity.

The selective antibacterial action, and the fact that activity could be demonstrated on a medium containing catalase, indicated that the active agent was a specific antibiotic substance and not hydrogen peroxide (Jennings and Sharp, 1947). This was confirmed in the case of the active substance produced in liquid media, culture fluids and concentrates derived from them both exhibiting equal activity with and without the addition of blood as a source of catalase.

The antibacterial action of the concentrated active substance, freed from living bacteria, was investigated qualitatively and quantitatively by the gutter-plate method (Fleming, 1929) and by dilution tests.

Gutter-plate tests.

In these the antibiotic preparation, in various amounts, was incorporated under sterile conditions in a strip across the diameter of an agar plate and various test organisms were streaked across the plate at right angles to it.

A solution of trichloracetic acid-precipitated material which had been redissolved in one-twentieth of the volume of original culture fluid, and which gave zones of inhibition against *C. xerosis* by the cylinder-plate method of 21·5 mm. diameter undiluted and of 11 mm. when diluted 1 in 64, was incorporated in the gutters at dilutions of 1 in 2 and 1 in 4. Inhibition of the following organisms occurred, the distance in millimetres from the edge of the gutter to the beginning of growth of the streak being shown by the figures in brackets. Each figure is the mean of 4 observations, that for the stronger solution being shown first. *M. lysodeikticus* (12, 12); *Myco. phlei* (8·5, 6); *C. xerosis* (8, 6); *Staph. aureus,*

NCTC No. 6571 (4, 2); and *B. anthracis* (4, 1). Organisms tested but not inhibited at these concentrations were *C. diphtheriae* (types *gravis* and *intermedius*) and *Str. pyogenes*, which were tested on serum agar plates; also *Bact. coli*, *Sh. sonnei*, *Ps. pyocyanea*, *Salm. gaertneri* and *Myco. smegmatis*.

With higher concentrations inhibition of *C. diphtheriae* could be demonstrated, solutions of dried active material containing 1, 2, 4 and 8 mg. per ml. and giving zones of inhibition against *C. xerosis* in cylinder-plate tests of 20 mm., 21·5 mm., 23·5 mm. and 25 mm. diameter respectively, were mixed with one-tenth of their volume of serum and one-fourth of their volume of melted 6 per cent agar and the mixture was filled into gutters.

Table I lists the inhibitions recorded.

TABLE I.—*Activity against Various Organisms as Indicated by Gutter-plate Tests.*

Concentration in gutter. Mg. per ml.	Distance (mm.) from edge of gutter to which inhibition extended.			
	Bact. coli.	*C. xerosis.*	*C. diphtheriae (gravis).*	*Staph. aureus.*
0·7	0*	6·0	5·0	3·5
1·4	1·5	5·0	7·0	4·0
3·0	2·5	6·5	8·0	5·5
6·0	3·5	8·0	8·5	6·5

* Growth over the gutter itself was inhibited, however.

Comparative tests were carried out with solutions of active material precipitated by trichloracetic acid from liquid cultures and from an extract of solid medium on which the organism had grown, in order to ascertain whether the latter contained any additional active substances which were not being produced in liquid medium. This did not appear to be so, as both solutions gave similar zones of inhibition of plates seeded with *C. xerosis* and were found by the gutter-plate method to act in an identical manner on *C. xerosis* and on *C. diphtheriae* (*gravis*), the former organism being the more sensitive. Solutions which had been sterilized by filtration through sintered glass proved to be less active than those which had been immersed in boiling water for 2 minutes.

Dilution tests in liquid media.

These were carried out on a dialysed solution of the active substance which had been precipitated by ammonium sulphate from a solution of lyophilized trichloracetic acid-precipitated material. After heating to precipitate coagulable material and removing the latter by centrifuging, the solution contained 13·2 mg. of solids per ml. (1 part in 77). It was sterilized by immersion for 3 minutes in a boiling water bath. By the cylinder-plate method it gave zones of inhibition against *C. xerosis* of 26 mm. undiluted and 14 mm. at a dilution of 1 in 64 (1 part in 5000).

Two-fold dilutions were made, commencing at 1 part in 770. The test was carried out with a series of organisms, the inoculum being one drop per tube (measured by Dreyer's pipette) of overnight broth cultures which were diluted as shown in Table II. Ten per cent serum was included in the case of *C. diphtheriae* and *Str. pyogenes*. The results were read after 16 hours at 37° C. and are set out in Table II. In no case was the degree of activity of a high order.

TABLE II.—*Activity Against Various Organisms as Indicated by Dilution Tests in Liquid Medium.*

Test organism.	Inoculum; broth culture diluted 1 part in:	Inhibited completely at 1 part in:	Inhibited partially at 1 part in:
Staph. aureus NCTC 6571	1000	6000	—
C. xerosis	100	2800	—
B. anthracis	100	2800	—
Str. pyogenes	10	1500	—
C. diphtheriae (*gravis*)	10	—	770

SUMMARY.

The production and properties of an antibiotic formed by a strain of *Staph. aureus* have been described. The substance was produced in shallow layers of liquid medium when an atmosphere rich in oxygen was maintained over the surface, but was not detected when air or oxygen was bubbled through the medium.

The antibiotic appeared to be a protein which could be precipitated from the cell-free culture medium by trichloracetic acid; it could be freed from the latter by dialysis and dried from the frozen state. It was relatively stable to heat in neutral or acid solution, but was rapidly destroyed by alkali, organic solvents, pepsin or trypsin.

The substance inhibited the growth of *C. xerosis*, *Staph. aureus*, *C. diphtheriae*, *B. anthracis*, *Myco. phlei* and *M. lysodeikticus*. In no case was the degree of activity, as measured by dilution tests in liquid media, of a high order.

The properties of the antibiotic are such as to make it probable that it will have no therapeutic application.

The author wishes to thank Prof. Sir Howard W. Florey and Dr. N. G. Heatley for help and guidance throughout the course of the work and in the preparation of this paper; also Dr. E. P. Abraham for reading the manuscript. Dr. M. A Jennings kindly arranged for the dilution tests which were carried out by Miss M. Lancaster and Miss M. Bond. The culture apparatus was designed and assembled by Mr. J. Kent, to whom thanks are also due for much assistance in its operation and in making the photograph.

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ELECTRON-MICROSCOPICAL STUDIES ON THE MECHANISM OF LYSIS AND THE MULTIPLICATION OF BACTERIOPHAGE OF *BACT. COLI*.

K. B. E. MERLING.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W. 1.

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OBSERVATIONS of living specimens with the ultramicroscope, using visible light, have given valuable information on the mechanism of lysis due to the action of bacteriophage of *Bact. coli* (Merling, 1938). By growing the bacilli in the presence of phage in hanging-drop cultures on the warm stage of the microscope it was possible to observe in dark ground illumination the process of lysis from beginning to end. The results were briefly that within 20 minutes of contact individual bacilli lost their motility and their normal shape and size, swelled, in most cases at one end more than the other, sometimes becoming spherical, and then suddenly burst, releasing the cell contents, which presumably consisted of bacterial protoplasm and masses of phage bodies. Counts showed about 250 phage bodies per lysed bacillus. It was assumed that the multiplication of the phage took place *inside* the bacterial cell, though proof for this assumption was lacking, owing to the limits imposed by the optical microscope

Since then a great amount of work has been carried out by a number of workers on the same subject. H. Ruska (1940), and later Pfankuch and Kausche (1940), described the tadpole shape of bacteriophage, and therewith established means of identifying phage bodies by their characteristic shape and size. Recently Wyckoff (1948) showed electron photographs in which he described " multiplication centres " and " foci," and made the statement that " apparently most of this growth and multiplication takes place in the protoplasmic masses liberated by lysis, rather than in bacteria which are still intact."

The experiments described here were performed to find how and where multiplication takes place, whether inside or outside the cell, and how many stages can be observed in the formation of the number of phage bodies that are seen on lysis and released by the bursting of one individual bacterial cell.

The problems proposed for investigation were : (1) Is it possible to correlate the findings of observations on living cells, showing that between 200 and 250 phage bodies were released from one lysed bacillus with the pictures obtained in the electron microscope ? (2) How and where does the multiplication of the bacteriophage of *Bact. coli* take place ?

MATERIAL.

The strains of *Bact. coli* and bacteriophage were the same as used previously for living observations. Twenty-four-hour broth cultures of *Bact. coli* were plated on agar, and broth containing phage was added in single drops to the

surface-dried plate following the usual technique. After incubation for 6, 12, 24 and 36 hours, specimens were prepared from the edge of plaques and other parts of the plaques and plate. By taking specimens at increasing distances from a single plaque in the centre of a plate culture, earlier stages of lysis could be found than in samples taken simultaneously from the plaque edge. Other specimens were prepared by making drop cultures of *Bact. coli* and bacteriophage on the membraned grid of the electron microscope, and were examined at times of contact varying between 1 and 45 minutes. In these specimens the material had to be mounted in doubly distilled water, after experience had shown that broth suspensions were unsuitable for examination in the electron microscope, and further that accurate timing of contact was largely impossible owing to the lag introduced by the repeated washing necessary to remove salts from the specimen. The timing of contact and process of lysis was carried out by removing the meniscus of the drop cultures by means of micropipettes, a process which took no longer than 2 or 3 seconds.

TECHNIQUE.

Specimens were prepared for examination in the electron microscope in the usual way by mounting collodion membranes on copper grids. The collodion membranes were prepared by adding to the usual solution of 2 per cent pyroxilin in amyl acetate, 3·5 per cent of ethyl lactate as a plasticizer. This was suggested by Dr. A. Burgen as a means of obtaining membranes both thinner and of greater resistance to heat and electron bombardment than the ordinary collodion film. The membranes containing this plasticizer were highly successful not only when used immediately, but after drying in a desiccator for several days.

To increase contrast the shadow-casting technique, invented by Wyckoff and Williams (1945), was used. The amount of metal evaporated and coated on to the specimen, however, was kept at a minimum to avoid distortion; the aim was to increase contrast, not the development of shadows, as it was not intended to obtain measurements in this way.

Coating was carried out in a specially designed apparatus, made by Metropolitan-Vickers, which was inserted in the specimen chamber of the electron microscope. As coating materials gold, palladium and gold-palladium-alloy 40 : 60 were used.

Hanging drop cultures were also made of all material prepared for the electron microscope, and kept under observation in the living state on the warm stage of the ultra-microscope. These specimens served as controls to safeguard against misinterpretation, and to determine the exact state of the electron microscope specimen at the moment of drying.

Observation in the electron microscope was greatly facilitated and improved by the introduction of a fluorescent screen (Merling, 1949) made of zinc-cadmium-sulphide by Derby and Co., London, E.C.2, which together with other characteristics has a $2\frac{1}{2}$–$3\frac{1}{2}$ times greater brightness than the usual Willemite screens.

RESULTS.

The study of bacteriophage is made difficult by the speed with which lysis of an individual bacterial cell is completed, and is further complicated by the fact

that the speed of lysis for individual cells varies from about 5 minutes to about 20 minutes after first contact of the bacterial cell with bacteriophage.

Normal bacilli.

To detect small changes in the appearance of the bacilli it was, of course, necessary to become thoroughly familiar with the appearance in the electron microscope of " normal " bacilli at as many stages of their development as possible.

From the experience gained in this way a composite picture has been gained which shows that a " normal " bacillus during its life goes through a definite cycle. It begins as a short rod of about 0·8 or 0·9 μ in breadth, and varying in length from 1·2 to 3 μ. This rod in the course of 12 hours either develops intracellular divisions which will eventually progress until separation of the daughter cells is complete, or it may deteriorate, showing granules of varying size, in which state it may be dying or dead. The very young forms, in cultures up to 12 hours old, may show very little differentiation (Fig. 1), and their internal structure is restricted to shadowy areas without sharp contours. The surface layer or membrane of the bacillus can be distinguished from the internal structure by its lower scattering power for electrons, and shows as an envelope of low brightness, which in the perspective of the electron microscope appears to have a width of about 10 per cent of the breadth of the bacillus.

While young forms may as a rule be regarded as mainly structureless, there are conditions, so far not determined, under which the seemingly " normal " bacilli exhibit areas of greater density, particularly at the poles, and in one or two other places. Fig. 2 shows two such cells, one with polar bodies and two more spots of higher scattering power, the other probably distorted in the fortuitous perspective owing to the fast drying of the specimen.

Twenty-four-hour cultures show the beginning of differentiation usually in that the greater part of the cell contents appear to contract away from the poles of the bacilli, and generally towards the central axis of the cells, thus leaving the poles more or less empty and transparent. The bacterial membrane then becomes very clearly visible and shows a structure consisting of two parts, one coarse, appearing like a branching system extending from the central axis, the other being the envelope or membrane proper (Fig. 3). Bacilli from cultures more than 24 hours old show the progressive development of more or less well defined internal structures. This differentiation, as mentioned above, may be an indication of approaching death (Fig. 4 and 5).

Whilst the three appearances described successively dominate the picture according to the age of the culture, it must be emphasized that, whilst at any given moment the majority show one appearance, cells can also be found at other stages of development.

It has also been possible to correlate the appearances seen in the electron microscope with those observed on living cells in the ultra-microscope. Although with the ultra-microscope it is difficult to see internal structures in the living bacilli before they have reached the state of granularity, it is just possible to glean indications that structures are present, with special methods such as the use of immersion media of a refractive index equal to or near that of the bacterial membrane (Merling, 1935).

Normal bacilli in the presence of bacteriophage.

It is known from observations on living material that the first visible result of contact between a bacillus and bacteriophage is the loss of motility of the bacillus. The flagella, which normally are quite active, become motionless within a few minutes of contact and the bacillus lies still. It has, however, been very difficult to observe a bacillus in this state in specimens prepared for examination in the electron microscope.

Fig. 6 shows a bacillus with some phage bodies adsorbed, but it is impossible to say whether or not one has actually entered the bacillus. The protoplasm of the bacillus is receding from the membrane unevenly.

Fig. 7 shows a bacillus from a 6-hour culture which has had two minutes, contact with bacteriophage. Within the two minutes seven phage bodies have been adsorbed on the surface of the bacillus and one of them has entered the cell, where it can be seen nearly surrounded by the bacterial membrane, which has presumably formed a slight extrusion, indicating entry by a foreign body ; in the same picture is a small cluster of phage bodies.

Fig. 8 shows a bacillus which differs from the " normal " only in so far as the bacillary protoplasm has receded from the membrane evenly all round. The head of one phage body has penetrated the bacillary membrane which is beginning to form an extrusion around it.

In Fig. 9 the entry of one phage body into a slightly older bacillus is shown. The bacillary membrane has not yet completely engulfed the phage body, which is already dividing into two phage bodies.

Fig. 10 is another example of one phage body inside an extrusion of the bacterial cell. This is shown on the left of the figure underneath a small cluster of phage bodies.

It has not been possible to correlate the behaviour of the flagella as seen in the living state with the picture in the electron microscope. All that can be said at the moment is that in a lysing culture there appear to be more free flagella than in the non-lysing controls, a fact which is known from observations on living cells.

Bacilli infected by bacteriophage.

At the moment of contact between bacillus and bacteriophage the bacillus ceases to be normal, although with the present technique changes in appearance become detectable only when multiplication of phage has started within the bacillus. Two types of changes in appearances can then be distinguished : (*a*) those due to the ageing of the culture ; (*b*) those due to the presence of bacteriophage and the consequent progressive lysis. These two types of appearances become superimposed.

The first stage in the multiplication of the bacteriophage appears to take place at the point of entry as shown in Fig. 10.

Fig. 11 shows the point of entry with the extrusion from the bacterial membrane enveloping a phage body in simple division. Two phage bodies are attached to the surface of the bacillus.

When contact between phage and bacilli is maintained for longer than five minutes at 37° C. the first and second stages, of entry and multiplication at the point of entry, are accompanied by further stages of development. This indicates that newly grown bacilli are continually being infected, and lysis progresses with great rapidity.

Fig. 12 shows a small area of greater density in the otherwise clear pole of the bacillus, which appears to be a centre of multiplication or the beginning of an intracellular colony after the entry of one phage body, although details of that multiplication cannot be discerned.

In Fig. 10 the bacillus, of which the greater part is shown, has two round masses at one pole, which have some structure and differ in that respect from polar bodies and other normal cell constituents.

Fig. 13 shows a bacillus with similar masses in which the centres are becoming slightly transparent, and Fig. 14 shows several such round bodies coalescing and becoming clearer in outline. It will be necessary to refer to these appearances later.

In order to gain a picture of the further multiplication of bacteriophage various procedures were tried. First of all it appeared promising to wash the bacilli in double distilled water in order to remove salts. This was done after preparing the specimen in the usual way on the grid. After one to five minutes' contact at 37° C. the meniscus containing the specimen was pipetted off, and distilled water at room temperature was applied with another micropipette and sucked off, this being repeated three times. The specimen was then transferred to a small desiccator, and dried quickly over phosphorus pentoxide by a water pump.

This procedure did not have the desired effect of removing salts to any great extent. On the other hand, it produced in some cells something that can only be regarded as an artefact, although it may be considered as similar to selective staining in light microscopy. Apart from the affected cells (Fig. 15–18) there were always great numbers of cells similar to those of unwashed specimens. It is not known what governed this selection.

The most outstanding feature presented by the affected cells (Fig. 15–18) is the granularity of the bacilli. On closer inspection it can easily be seen that there are two types of granules of distinctly different size, (*a*) granules averaging about 500 Å and (*b*) granules about 200 Å in diameter.

Fig. 15 shows a bacillus with some fairly well-defined large spherical structures inside, together with granules of the smaller type. Attached to the bacillus, or accidentally positioned near it, is a spherical mass which may be interpreted either as a cell rounded up prior to bursting, or as a large extrusion from the adjacent bacillus containing a colony of phage.

Fig. 16 is another example of similar appearances showing granules of the larger type. The arrangement of the granules is well shown and, although there seems to be mainly a random distribution, at some points semi-circular grouping can be seen.

Fig. 17 shows a cell with large type granules, in which part-circular arrangements of the granules can also be discerned.

Fig. 18 shows small type granules and contraction of the protoplasm of the cell towards one pole and one side of the cell, as well as some chromatin globules. At the left pole of the same cell, in which there is no polar body, there are about 25 large type granules showing concentric semicircular arrangement.

Apart from granularity another effect shown as a result of repeated washing by nearly all the figures of such specimens (Fig. 16–18) is the disappearance of the bacterial envelope. These effects will be considered in the discussion.

The third stage of lysis consists of the disruption of the bacterial cell and the release of its contents.

Fig. 19 shows the outflow of the contents of a cell which has just burst at one end. Flagella, phage bodies and bacterial protoplasm can be discerned.

In Fig. 20 a cell is also disrupting from one end, whilst the other end, not shown in the photograph, appears to be still intact. A few phage bodies can be seen in the midst of rather coarse granules of bacillary protoplasm.

Fig. 21 shows the bacterial protoplasm dispersing from a cell, of which only the densest parts, i.e. polar bodies, etc., persist. Lysis seems to proceed by disruption of the whole cell with complete disappearance of the cell membrane. This picture compares well with the appearances seen in the living specimen in the ultra-microscope. Great numbers of phage bodies can be seen in the dispersing bacterial protoplasm, which seem to consist of droplets with well-defined surfaces giving the appearance of a foam among which the phage bodies are interspersed.

Fig. 22 shows this bacterial protoplasm together with phage bodies adhering to it. During the disruption of this cell the contents have spread out rather more than usual, so that two negatives have had to be used to make this figure. In the left upper corner can be seen a part-circular arrangement of phage bodies.

The disruption of the lysed cell may take any form, varying with local conditions in the immediate vicinity of the specimen.

In Fig. 23 is shown part of a lysed cell in which a few phage bodies are still retained. This figure shows that the phage bodies have a tail prior to dissociation from the lysed cell. The tail cannot always be seen under such conditions owing to the very much reduced resolution brought about by the overlying protoplasm, which produces additional scattering of the electrons and therewith reduces contrast. It can easily be seen that if the resolution demanded is in the neighbourhood of 50 Å and structures separated by 50 Å are supported or overlain by a film, be it collodion or protoplasm of equal thickness within the same dimensions, such resolution can be obtained only under exceptional circumstances.

Attention has already been drawn to the part-circular arrangement of phage bodies, of which a few more examples are given in Fig. 24 and 25, in both of which the droplet structure of the bacterial protoplasm is also apparent. Repeated observation of the part-circular grouping of phage bodies led to an attempt to discover the way in which the phages multiply.

In Fig. 10, 13 and 14 some round masses are shown inside bacilli of more or less intact appearances. In Fig. 26 a rounded-up cell is shown in which the contents appear as one mass bordered by 5 semicircles. This is interpreted as 5 colonies of phage coalescing in the centre.

The inclusion body or intracellular colony need not necessarily be circular or spherical. In Fig. 28 the phage bodies are of a slightly ovoid shape with no tails, and are arranged altogether in two three-quarter circles, enveloped by a membrane. These are regarded as two small colonies of phage which have been discharged in this state of development from a lysing cell. A similar aspect is shown in Fig. 29 and 30, in which, however, the resolution is greatly reduced owing to the presence of bacterial protoplasm.

The breaking up of the colonies seems to be represented in the appearance of large clusters as shown in Fig. 31, and smaller clusters as shown in Fig. 32. Fig. 33 and 34 are further evidence of this part-circular grouping of the phages.

Both figures exhibit low resolution owing to the presence of bacterial protoplasm.

The tracing of the multiplication of bacteriophage so far has left out one important link, that of the development of two phage bodies from one.

Fig. 35 shows three groups of phage bodies, one of which is a group of five in a part-circle arrangement the two others consist of pairs of phages. Closer scrutiny of these pairs shows the central pair to consist of two complete phage bodies with tails and divisions in the heads. The division of the other pair is not clear, and only one phage has a tail. The remarkable feature of this micrograph is the close proximity of the members of the pairs, so that they seem to be flattened against each other. The resolution in this figure is very high compared to that in Fig. 9 and 11, which show binary fission of phages still inside the cell. The interpretation suggested is that these pairs indeed represent phages in "binary fission." With this figure the evidence of multiplication is regarded as complete.

DISCUSSION.

Short survey of working conditions.

In any assessment of results obtained by means of the electron microscope it is necessary to appreciate the limitations as well as the advantages of the instrument.

The dehydration of the specimen in the electron microscope produces *a priori* conditions most unnatural for living organisms, and in this respect the electron microscope compares unfavourably with the light microscope. Radiation with light even when concentrated as in dark ground illumination can be rendered more or less harmless with suitable light sources and filters, whereas electron bombardment as used at present in the electron microscope cannot be varied to any great extent, apart from keeping its intensity down to the minimum necessary to make visible the structures to be observed, and to record them photographically. Parallel to this bombardment goes the heating of the specimen. Both these factors in addition to dehydration may alter the specimen.

When structures which are below the resolution limit of the light microscope are under observation in the electron microscope, controls in the light microscope

EXPLANATION OF FIGURES.

Bact. coli and bacteriophage. Magnification about × 18,000. Fig. 7, 10, 15, 19, 20 shadowed with gold, all others with gold-palladium alloy.

Fig. 11 reproduced from *Nature* by permission of the editors.

PLATE I.

FIG. 1.—Very young "normal" (12-hour culture) bacillus showing very little internal structure.

FIG. 2.—Two very young "normal" (12-hour culture) bacilli showing polar bodies and other structures.

FIG. 3.—Two bacilli from 24-hour culture showing increasing differentiation of internal structure and structure of membrane.

FIG. 4 and 5.—Bacilli from older culture (36-hour) showing granules of various sizes.

FIG. 6.—A bacillus whose protoplasm has receded unevenly from the membrane. Some phage bodies are adsorbed; one is possibly inside the bacillus. Contact 3 minutes.

FIG. 7.—Bacillus (6-hour culture) after two minutes' contact with bacteriophage. Seven phage bodies have been adsorbed on to the surface of the bacillus; one phage body has entered the cell and is surrounded by the bacterial membrane. Flagella in great numbers. Contact 2 minutes.

FIG. 8.—A bacillus forming an extrusion around one phage body. Its protoplasm has receded slightly but evenly from the membrane. Contact 5 minutes.

FIG. 9.—Entry of one phage body into one bacillus. The extrusion from the bacillary membrane has not completely surrounded the phage body, but this has already divided into two. Contact 5 minutes.

Plate II.

Fig. 10.—Parts of two bacilli. Between them a small cluster of phage bodies. One phage body inside an extrusion of the bacterial cell on the top. The lower bacillus shows two round masses in the pole. Contact 10 minutes.

Fig. 11.—A bacillus with (on the top) two phage bodies attached to its surface and (on the lower left) an extrusion enveloping a clearly visible phage body in simple division. One thrown-off phage-tail lies opposite the extrusion. Contact 8 minutes.

Fig. 12.—A small area of greater density in the otherwise clear pole of the bacillus is a centre of multiplication after the entry of one phage body. Contact 5 minutes.

Fig. 13.—A bacillus with two areas whose centres are slightly transparent. These areas have some structure and differentiation which allows them to be distinguished from polar bodies and other normal cell constituents. They are regarded as intracellular colonies of phage. Contact 15 minutes.

Fig. 14.—A bacillus showing these areas coalescing. From plaque edge.

Fig. 15–18 are taken from specimens that have been washed three times with doubly distilled water.

Fig. 15.—A bacillus with small granules and a number of spherical areas of greater density, and attached to the bacillus a spherical mass which may be either a rounded-up cell prior to bursting or a large extrusion from the adjacent bacillus containing a colony of phages. From plaque edge.

Fig. 16.—Two cells superimposed. High resolution at the edge of the cells shows the part-circular arrangement of the granules. From plaque edge.

Fig. 17.—Cell with large type granules in part circular arrangements. The polar bodies seem to be disappearing. From plaque edge.

Fig. 18.—Cell with some small-type granules in concentric semicircular arrangement. From plaque edge.

Plate III.

Fig. 19.—Pole of a cell in the process of bursting, discharging its contents of protoplasm and phage bodies. The beaded appearance of the flagella is due to the coarse recrystallization of the gold coating. From plaque edge.

Fig. 20.—A similar picture to Fig. 19. In the bacillary protoplasm there are few phage bodies and large numbers of small type granules. From plaque edge.

Fig. 21.—Lysing cell, of which some parts are persisting whilst others disperse together with phage bodies. From plaque edge.

Fig. 22.—(Taken on two negatives.) Bacterial protoplasm of a lysed cell showing in the form of droplets to which numbers of phage bodies adhere. In the left upper corner the phages show part circular arrangement. From plaque edge.

Fig. 23.—Part of a lysed cell. The phages adhering to it have tails. Contact 30 minutes.

Fig. 24.—Part circular arrangement of phage bodies adhering to the protoplasm. Contact 50 minutes.

Fig. 25.—A similar picture of part circular arrangement of phage bodies and protoplasmic droplets. Contact 50 minutes.

Fig. 26.—A rounded-up cell whose membrane is faintly visible. The cell contents have formed one mass with five semicircular bulges. The edges of these bulges show greater density. Contact 1 hour.

Plate IV.

Fig. 27.—A lysed cell showing an elongated conglomeration of phage bodies. From plaque edge.

Fig. 28.—Two-part circular arrangements of phage bodies within a membrane: small colonies of phage discharged as a whole from a lysed cell. From plaque edge.

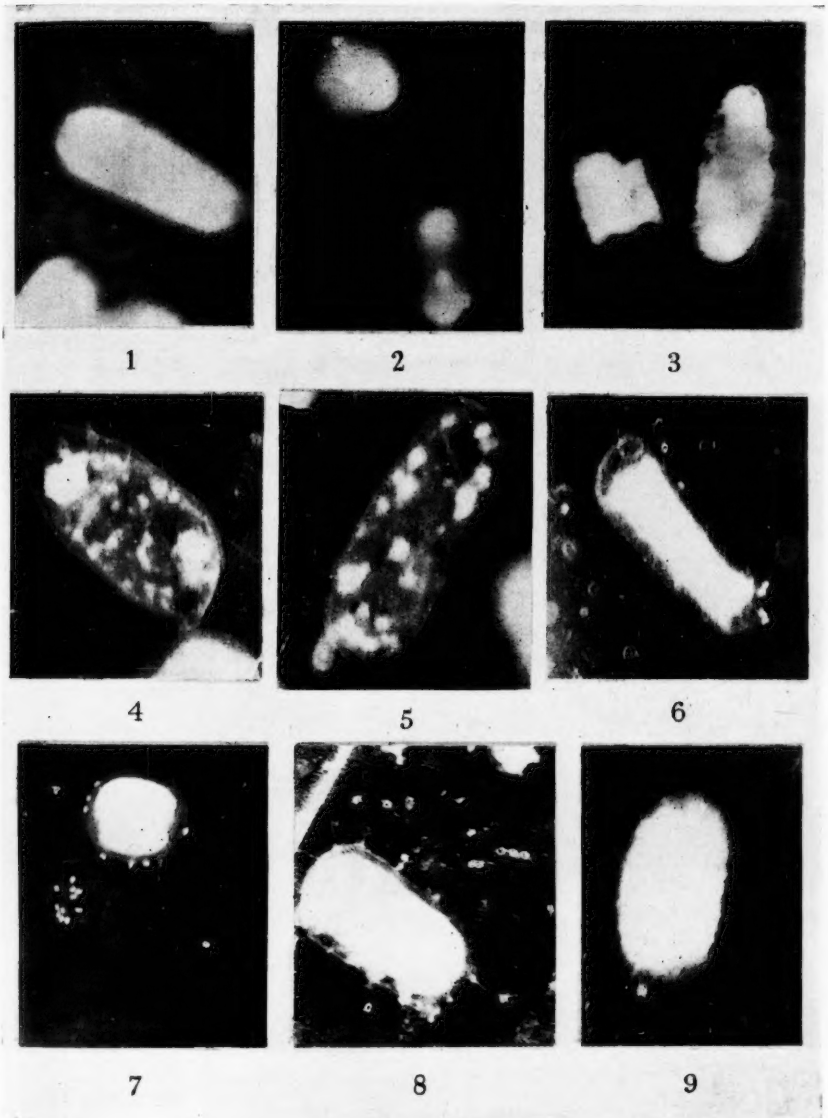
Fig. 29.—Very nearly circular arrangement of phage bodies within bacterial protoplasm. From plaque edge.

Fig. 30.—Similar to Fig. 29. Nearly circular arrangement of phage bodies. From plaque edge.

Fig. 31 and 32.—Large and small clusters of phage bodies breaking away from a previously circular or semicircular arrangement. From plaque edge.

Fig. 33 and 34.—Part-circular arrangements of phage bodies still inside bacterial protoplasm of cells just breaking up. Contact 1 hour.

Fig. 35.—Three groups of phage bodies, one group in part-circular arrangement, the other two consisting of pairs. Resolution in this figure is very high and allows of very close scrutiny, which reveals a remarkable closeness in the position of the members of these pairs, which seem to be flattened against each other: binary fission. Contact 1 hour.



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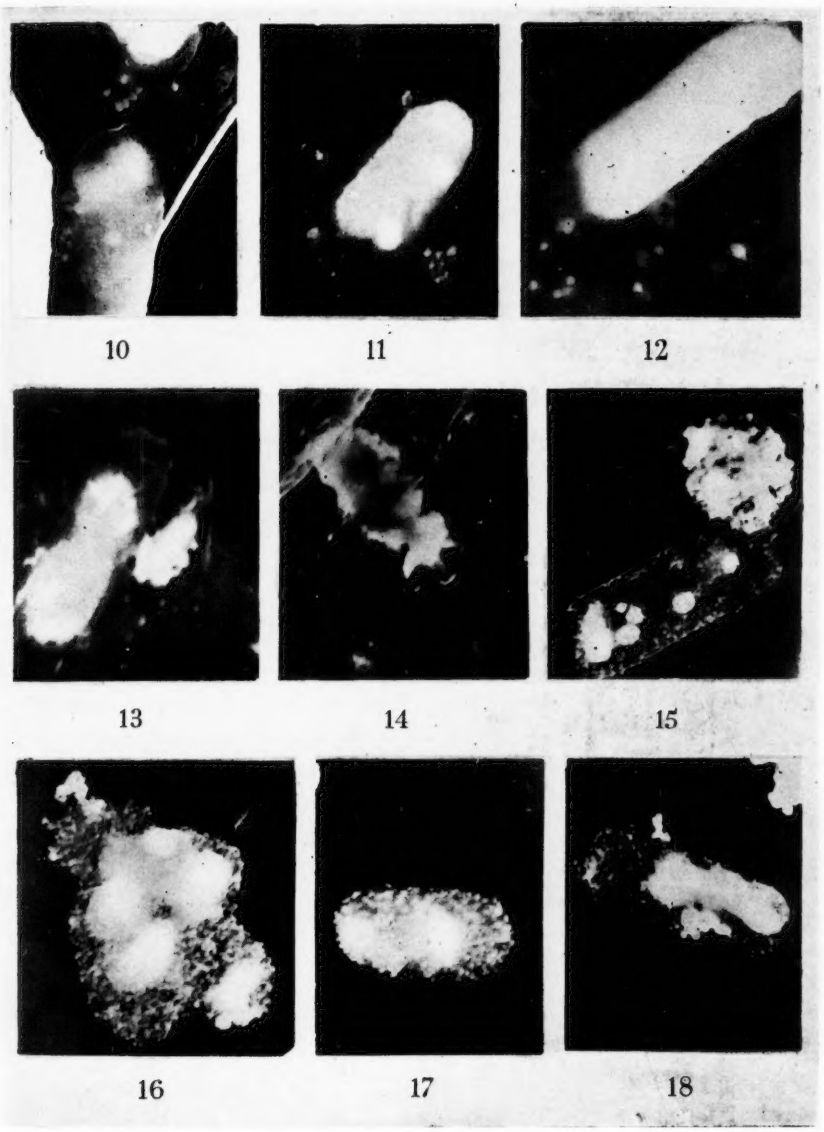

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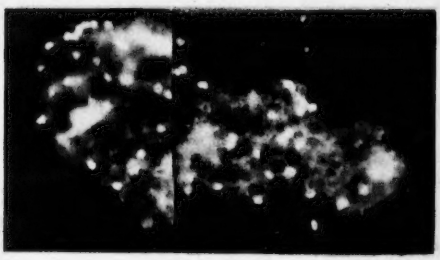

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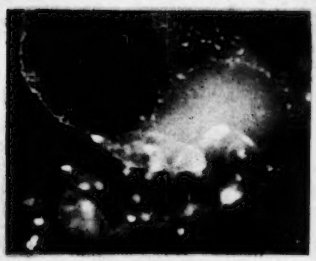

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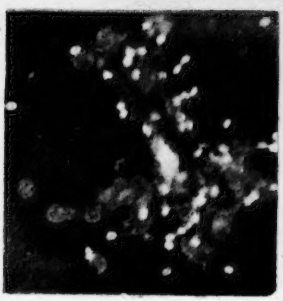

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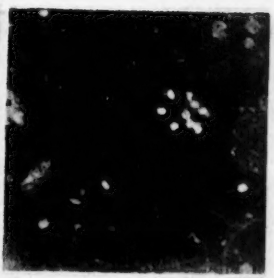

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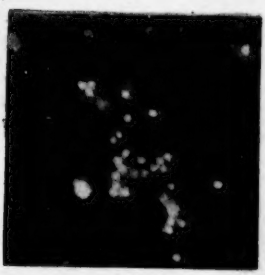

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become largely valueless. Such controls, however, have proved their worth in the study of structures that fall within, or even slightly beyond the known limits of the light microscope. It is at this point, the " limit of resolution " in the light microscope, where perhaps some progress is to be expected from knowledge gained by observations with the electron microscope.

Bacteriological specimens.

The loss of water which all specimens must suffer in the high vacuum of the electron microscope may have totally unpredictable results as far as biological objects are concerned. Some bacteria have been shown to be flattened out from their normal rod shape into flat ribbons ; others have kept their shape, as shown by their profiles and shadows. All-round shrinking seems to occur rarely. Very young forms thus may be small originally, as shown in the light microscope, or their small size may be due to a relatively greater water loss than occurs with older forms, which presumably possess a stronger envelope. Any statement with respect to the size and shape of micro-organisms and their parts must take those factors into account.

Electron bombardment and heat have very distinctive effects on the specimen. Observation for several minutes generally leads to loss of contrast. This is probably due to the deposit of tungsten oxide on the collodion membrane, which darkens the background. Heat may wrinkle and tear the membrane, though tears are usually due more to electron bombardment than to heat. A wrinkled and baked membrane is unsuitable for further examination. A specific effect of electron bombardment is the " building up " of fine structures into thick, coarse artefacts. The smaller the unit under observation the less will be the effect of dehydration, although heat and electron bombardment still exercise their distinct influences.

In the case of *Bact. coli* the drying has resulted in most cases in severe flattening of the bacterial cells, which has been apparent in profiles and shadows. On the other hand, the bacteriophage bodies do not show flattening, either in profile or by the shadowing method.

The picture presented by the electron microscope of " normal " bacilli in their ordinary development from the young uniformly filled cell to the granular appearance of the old form, corresponds closely to the well-known images seen with living bacilli in the light microscope. Minor difficulties of interpretation may arise from pictures showing old forms. What is called " granularity " comprises a multitude of appearances, of which only two examples (Fig. 4 and 5) are given here. In many cells two types of granules can be distinguished : (*a*) 2 or 3 large particles of about 0·3 μ in diameter, mostly situated in the poles of the bacillus, and (*b*) smaller particles of about 0·05–0·1 μ in diameter, of which there may be great numbers. In the electron microscope other cells may appear completely empty, or show only polar bodies of great density. The arrangement of the smaller particles may be similar to that shown by intracellular colonies of phages.

However, it is the age of the culture that determines the proportion in which these various types of cells appear. The impression has been gained from the examination of many hundreds of specimens that the life of the ordinary bacterial cell is speeded up in the presence of phage, resulting in quick ageing and death.

The first visible reaction of the majority of normal bacilli to the presence of

bacteriophage has been stated to be their loss of motility. In the electron microscope the flagella of *Bact. coli* are not seen regularly, and when visible appear to be mostly thrown off. At any rate it has not been possible to compare the behaviour of the flagella in " normal " cells and infected cells.

The first real contact between bacteria and phages appears to be a process of adsorption. It is difficult to decide whether only one phage body can enter into one bacterial cell. Delbrück (1945) has speculated upon this process, which he likens to the " fecundation of the monospermic egg." The only evidence in favour of the one phage body one bacillus relationship is the repeatedly observed fact that out of a number of adsorbed phages only one phage body per bacillus has been surrounded by an extrusion of the bacillary membrane at the time of observation (Fig. 7, 9 and 11). The formation of this extrusion around one phage body only may be another expression of the axiom " First come first served," first proposed by R. Salaman in the case of potato viruses X and Y, where infection with one virus excluded the other. It has not been possible to see anything of this process in the living specimen in the light microscope.

There are similar extrusions from " normal bacilli." Therefore the extrusion may be : (1) an effect of drying, (2) a sign of commencing disintegration, (3) a non-specific reaction due to local conditions, (4) a specific reaction to the entry of a foreign body, (5) a specific reaction to the entry of one phage body.

If the extrusion is specific and part of a mechanism preventing multiple entry, a surface alteration should take place in the structure of the membrane as a result of phage entry. On the other hand, the development of several intracellular colonies in one bacillus, while not excluding their origin from one phage entry, would suggest multiple infection.

That the dense intracellular masses may be phage colonies has also been suggested by Wyckoff, although he states that the greater part of growth and multiplication of the phage takes place in the discharged protoplasm of the dead bacterial cell. Only improved technique can decide whether this view is possibly correct. The present author is of the opinion that most of the phage growth and multiplication takes place within the bacterial cell before its disruption, and that the finding of phage bodies in clusters and adherent to discharged cytoplasm is due to a slowing down of the process of lysis during the quick drying procedure, which in many cases prevents the complete separation and dispersal of the phage bodies.

That the method of preparation of the specimen has a profound influence is shown by Fig. 15–18 of specimens that have been washed 3 times with distilled water. It appears that in the examples shown the water has had a distinct dissociative action upon the cell constituents. No explanation is offered for the frequent complete disappearance of the bacillary membrane. It is not yet known whether this granularity represents phage colonies together with an even distribution of phage bodies over the whole interior of the bacillus, but an answer to this question may yet be found with a different technique. The sizes of granules displayed within the cells suggest that liquefaction of some of the cell constituents has occurred owing to the presence of the intracellular phage colonies, which can then discharge the individual phage particles into the surrounding liquid. Fig. 16 and 17 are perhaps most suggestive of this explanation. The nature of these granules is uncertain because they are smaller than the phage bodies seen normally. It is likely that this liquefaction is an artefact brought about by

the distilled water. The selective action of this washing on some out of a great number of cells indicates a different reaction on the part of these cells. No normal cells have shown a similar or comparable appearance after the same treatment. It is the relatively high incidence of these granulated cells that justifies the inclusion of these appearances. The moment of disruption has been caught in Fig. 19 and 20. The number of phage bodies adherent to the discharged protoplasm of the lysed cell may vary considerably, there being large numbers in Fig. 21, 25 and small numbers in Fig. 22, 23 and 24. The greater the number of phage bodies adherent to remnants of the cell the greater is the chance of finding them in groups (Fig. 21). A frequent appearance in these specimens is that instanced in Fig. 26 and 27, where the interpretation of single or multiple masses as inclusion bodies, or colonies of phage, suggests itself when the grouping of phage bodies is confirmed in definite assemblies (Fig. 28, 29, 30, 33 and 34). Coalescing colonies (Fig. 26) can be recognized. The finer structure of the phage colony, persisting after the disruption of the bacillus, is shown in Fig. 28, 29 and 30. Fig. 28 also shows that the colony has a membrane or some sort of envelope enclosing it. The significance of colony formation by bacteriophage lies, of course, in the implication that only a living organism is capable of doing so. It is in full appreciation of this significance that the statements regarding intracellular colonies are made. It is comparatively rarely that fully transparent colonies can be found, as is only to be expected, because the resolution in a specimen must be reduced as soon as the overall thickness of the specimen exceeds a maximum value estimated at about 100 Å. Nevertheless, the grouping of the phage bodies emphasized repeatedly above can be seen easily enough. Fig. 33 and 34 are further examples of this particular arrangement of phage groups, which is regarded as an indication of the mode of multiplication, of which the first step is taken to be binary fission.

Wyckoff suggests that bacteriophages develop from smaller forms. If there is binary fission there ought to be no need to interpret smaller granules as smaller forms of development. Moreover, these smaller granules ought to be present in greater numbers than the fully grown phage bodies, particularly if lysis is interrupted at the right moment. This, however, appears not to be the case. On the other hand, appearances strongly suggesting binary fission can be shown both intracellularly and extracellularly (Fig. 11 and 35). Wyckoff further suggests as quoted above that " most of the bacteriophage growth takes place in the protoplasmic masses liberated by lysis," that is, after the death of the bacilli. In view of the colony formation described in this paper and experiments with other living viruses, the present author holds that the multiplication of bacteriophage takes place to its greatest amount within the bacterial cell before its disruption, although it is unlikely that the cell is still alive in the normal sense at the time of release of the virus. There is, however, a strong possibility that some phage bodies not yet fully developed at the moment of disruption of the cell will finish their development while still attached to some discharged bacterial protoplasm. This process can be likened to a clockwork running down, but is not regarded as the normal mode of multiplication of bacteriophage.

Smaller granules, similar to those shown in Fig. 16–18, have been seen in circular or part-circular arrangement similar to the colonies shown in this communication, but they are rather isolated and uncorrelated observations and their

significance can not be assessed. These observations are therefore not included in this publication.

It is suggested from the evidence produced by means of the electron microscope under control, as far as possible, of living observations in the light microscope that bacteriophage of *Bact. coli* is a living organism which, like other cell-parasitic viruses, forms intracellular colonies in which the single units of the phage multiply by binary fission.

SUMMARY.

Experiments were carried out with *Bact. coli* and its bacteriophage mainly by means of the electron microscope, but in conjunction with the ultramicroscope, in order to determine the mechanism of lysis and the multiplication of phage.

It was necessary to examine first the appearances shown by "normal" bacilli in the various stages of their development. This development was found to be speeded up in the presence of bacteriophage.

It was found that apparently the first sign of infection of the bacillus by a single phage body was an extrusion of the bacillary membrane engulfing one phage body whilst other phages might remain adsorbed to the surface. The second stage of the infection seemed to be the formation of intracellular colonies starting from the first entry and resulting in up to 6 such colonies to one bacillus. In the third stage, that of lysis, the bacillus burst either at one end or by disruption and disappearance of the bacillary membrane. In the discharged contents of the cell were found cell constituents like protoplasm, either as granules or as droplets, which might assume the appearance of a foam. Bacteriophage appeared to multiply by binary fission, which was seen either in freshly infected cells a few minutes after entry or in discharged clusters of phage bodies. Colonies, whether intracellular or discharged and persisting, showed a well-defined membrane.

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ANTIBIOTICS FROM PENICILLIA.

H. STOWAR BURTON.

From the Sir William Dunn School of Pathology, University of Oxford.

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MANY penicillia, aspergilli and basidiomycetes have been shown to produce antibacterial substances when grown under suitable conditions. A summary of the information produced by surveys in this field will shortly be published (Florey, Chain, Heatley, Jennings, Sanders, Abraham, Florey, 1949). This present paper records some results of a systematic attempt to fill in gaps in our knowledge of antibiotics known to be produced by fungi, but so far not further investigated. It is interesting in this respect to note that a high proportion of the penicillia examined produced already well known substances.

EXPERIMENTAL.

General conditions of culture.

All the penicillia investigated were grown in glass vessels on 500 ml. of medium 2 cm. in depth. Incubation was carried out at 24° C., the activity developing during culture being followed by the cylinder plate method of assay (Heatley, 1944).

Phoenicin from Penicillium rubrum.

The culture fluid of *P. rubrum* was reported to inhibit the growth of *Staph. aureus* by Miss M. E. Johns.

Conditions of culture.

The organism was grown on glucose Sabouraud medium containing 1 per cent peptone, 2·6 per cent malt extract and 4 per cent glucose for eight days.

Isolation.

The culture fluid at the time of harvesting was a reddish-purple colour. On acidification this colour disappeared, and the active principle was completely extracted from the acid solution into chloroform by shaking successively with one-half, one-quarter and one-eighth volumes of the solvent. The yellow chloroform solution was shaken three times with one-tenth of its volume of sodium bicarbonate solution, when the active principle passed to the aqueous phase, which became deep violet in colour.

The extraction into chloroform and re-extraction with sodium bicarbonate was repeated, using smaller volumes of solvent, in order to effect some purification. Final purification was effected by alumina chromatography. The final chloroform solution was evaporated, and the resulting yellow-brown crystalline material was recrystallized from ethanol.

Chemical properties.

The active substance crystallized in plates, m.p. 229–230° C. Found: C 58·61, H 4·17, CH_3(C), 9·7 per cent, active H 1·03 per cent. $C_{14}H_{10}O_6$ requires: C 60·5, H 4·02, CH_3(C) 10·9 per cent. It was slightly soluble in water, but soluble in chloroform, acetic acid and warm ethanol.

In aqueous solution it liberated iodine from acid potassium iodide, and it was reduced to a colourless compound by sodium hydrosulphite.

The substance appeared to be identical with the diquinone phoenicin, a compound isolated from *P. phoenicium* v. Beyma by Friedheim (1938) and examined by Posternak (1938). Phoenicin was reported to have the molecular formula $C_{14}H_{10}O_6$ and to melt at 230° C.

Antagonistic properties.

The crude culture fluid showed activity against *Staph. aureus*, *C. xerosis* and *Myco. phlei*.

Only a few milligrammes of the antibiotic were available for examination. At a dilution of 1 : 1000 it gave a zone of inhibition 22 mm. in diameter when tested by the cylinder plate method against *Staph. aureus*.

Expansine from Penicillium equinum.

The culture fluid of *P. equinum* was reported to inhibit the growth of *Staph. aureus* and *Bact. coli* by Miss M. E. Johns.

Conditions of culture.

The fungus was grown on modified Czapek-Dox medium containing 0·3 per cent sodium nitrate, 0·1 per cent potassium dihydrogen phosphate, 0·05 per cent potassium chloride, 0·05 per cent magnesium sulphate $MgSO_4 . 7H_2O$, 0·001 per cent of hydrated ferrous sulphate $FeSO_4 . 7H_2O$ and 4 per cent of glucose. Activity was maximal after about 10–15 days.

Isolation.

The culture fluid was shaken with $2\frac{1}{4}$ per cent acid charcoal and the active principle eluted from the latter with acetone. After distilling off most of the acetone the remaining fluid was passed through a short alumina column (pH 5), which removed much of the pigment but none of the activity. The antibiotic was extracted from the percolate with ether, and on evaporation of the ether an impure crystalline mass remained which was recrystallized from hot benzene or chloroform, giving pure colourless crystals.

Chemical properties.

After two recrystallizations the crystals melted at 111° C. On admixture with a sample of expansine (m.p. 111° C.) the m.p. of the mixture was 111° C.

When a solution of the crystalline material was warmed with 0·1 N sodium hydroxide solution a bright yellow colour developed. After neutralizing the alkaline solution the addition of aqueous ferrous chloride gave a deep purple-red coloration. The original aqueous solution gave no colour with ferric chloride solution.

The substance gave a yellow precipitate with 2:4-dinitrophenylhydrazine in aqueous 2-N-hydrochloric acid which after recrystallization from methyl alcohol melted at 216° C. (decomp.). The corresponding product from an authentic sample of expansine melted at 216° C. The substance gave an orange precipitate with phenylhydrazine hydrochloride which after recrystallization from hot water melted at 152° C. The corresponding product from expansine had the same melting point (Raistrick, Birkinshaw, Bracken and Michael, 1943).

Expansine from Penicillium novae zeelandiae.

The culture fluid of *P. novae zeelandiae* was reported to inhibit the growth of *Staph. aureus* and *Bact. coli* by Miss M. E. Johns.

Conditions of culture.

The fungus was grown on glucose Sabouraud medium containing 1 per cent peptone, 2·4 per cent malt extract and 4 per cent glucose. Activity was maximal at about the tenth day.

Isolation.

The active principle was removed from the culture fluid at pH 4 by continuous extraction with ether. On evaporation of the ether impure crystals were obtained. These were purified by recrystallization from hot benzene.

Chemical properties.

The substance melted at 110° C. The mixed melting point with a sample of expansine of m.p. 111° C. was 110°C. On warming a solution of the substance with 0·1 N sodium hydroxide solution a bright yellow colour developed. After neutralizing the alkaline solution addition of aqueous ferric chloride gave a deep purple-red coloration. The original aqueous solution gave no colour with ferric chloride solution. With phenyl-hydrazine hydrochloride the substance gave an orange precipitate, which after crystallization from hot water melted at 152° C. With respect to all these properties the substance was identical with expansine.

Mycophenolic Acid from Penicillium viridicatum.

The culture fluid of *P. viridicatum* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus.*

Conditions of culture.

The fungus was grown on the modified Czapek-Dox medium used for *P. equinum* containing 5 per cent neutralized corn steep liquor. Activity was maximal at about the seventh to the tenth day.

Isolation.

The active substance was removed from the acidified culture fluid by continuous extraction with ether. The brown gum which remained on evaporating the ether was dissolved in ethanol, and the potassium salt of the substance was precipitated by the addition of ethanolic potassium hydroxide. After centrifugation the precipitate was washed with ethanol, dissolved in water, and the free acid then precipitated by the addition of 2 N sulphuric acid. The precipitate was recrystallized from boiling water, giving small needle crystals.

Chemical properties.

The compound melted at 141° C. On admixture with an authentic sample of mycophenolic acid no depression in melting point was observed.

The acetyl derivative was formed when 0·25 g. substance was refluxed in an oil-bath at 120° C. with 2 g. acetic anhydride and 1 g. anhydrous sodium acetate. The mixture was poured into water, when the derivative formed an insoluble precipitate which was recrystallized from glacial acetic acid. M.p. 161° C. Mixed melting point with authentic acetyl mycophenolic acid 161° C. The compound gave a blue violet colour with ferric chloride in aqueous solution and a blue colour in alcohol.

Mycophenolic Acid from Penicillium bialowiezense.

The culture fluid of *P. bialowiezense* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus*.

Conditions of culture.

The fungus was grown on glucose Sabouraud medium containing 1 per cent peptone, 2·6 per cent malt extract and 4 per cent. glucose. Activity was maximal between the twentieth and twenty-fifth days, when the culture fluid was harvested.

Isolation.

The culture fluid contained a penicillin-like antibiotic which was destroyed by incubation of the culture fluid for two hours at 37° C. with penicillinase. The presence of this substance, presumably a penicillin, was responsible for about a third of the total activity. After incubation with penicillinase the culture fluid was extracted with ether at pH 3, which removed the residual active substance. The ethereal solution was concentrated and washed three times with one twentieth of its volume of saturated sodium bicarbonate solution. The main bulk of the active principle was extracted into 0·5 N sodium hydroxide solution. On addition

of hydrochloric acid to the resultant aqueous solution a white precipitate formed, which crystallized readily on scratching the vessel with a glass rod. The substance was recrystallized from hot benzene, hot water, or preferably from 4 per cent aqueous alcohol, when small colourless needles were obtained.

Chemical properties.

The compound melted at 141° C., and on admixture with an authentic sample of mycophenolic acid no depression of m.p. was observed. Found: C 63·7, 64·0, H 6·04, 6·6, CH_3(C) 7·1 per cent, OCH_3 7·95 per cent. Active H 0·46 per cent, M.W. (Camphor) 354, 359, 360, (Barger) 314. $C_{17}H_{20}O_6$ requires: C 63·7, H 6·3, M.W. 320.

The acetyl derivative was prepared as in the previous example and melted at 161° C. Upon admixture with an authentic sample of acetyl mycophenolic acid no depression in m.p. was observed. Found: C 62·5, H 6·13. $C_{19}H_{23}O_7$ requires: C 62·4, H 6·07. Barer, Cole and Thompson (1949) reported that the infra-red spectrum of the antibiotic isolated was identical with that of the authentic sample of mycophenolic acid.

Penicillic Acid from Penicillium baarnense.

The culture fluid of *P. baarnense* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus*.

Conditions of culture.

The fungus was grown on a modified Czapek-Dox medium, as used for *P. equinum*, together with 5 per cent neutralized corn steep liquor. Activity was maximal in 10 to 15 days.

Isolation.

Incubation with penicillinase destroyed about one-third of the total activity of the culture fluid. The activity of the residual material was not appreciably diminished in the presence of catalase.

After incubation with penicillinase, continuous extraction of the culture fluid at pH 2 with ether and evaporation of the ether yielded an oily material from which impure orange-coloured crystals were obtained.

The material was passed through an acid-washed alumina column (pH 5), when an active fraction was obtained which yielded almost colourless crystals.

Chemical properties.

The crystalline material was recrystallized from hot water, yielding crystals melting at 65° C. After recrystallization from petroleum ether the substance melted at 84° C. It was found that specimens of penicillic acid from *P. baarnense*, *A. quercinus* and *A. melleus*, partly melted at 38° C. when crystallized from water, ethanol or ether. The low-melting compound was a hydrate which was dehydrated in a vacuum desiccator over phosphorus pentoxide to give the non-hydrated penicillic acid m.p. 84° C. Found: C 56·4, H 6·4. $C_8H_{10}O_4$ requires: C 56·4 per cent, H 5·89 per cent. With ammonium hydroxide

a marked pink colour characteristic of penicillic acid developed, and upon admixture of the crystals with an authentic sample of penicillic acid no depression in melting point occurred.

With phenylhydrazine the substance gave a yellow crystalline derivative, m.p. 171° C.

The corresponding derivative from penicillic acid has been reported to melt at 171° C. and 175° C. (Alsberg and Black, 1913; Birkinshaw, Oxford and Raistrick, 1936).

Herquein from Penicillium herquei.

The culture fluid of *P. herquei* was reported by Miss M. E. Johns to inhibit the growth of *Staph. aureus*. The antibacterial substance in the fluid has been isolated and named herquein.

Conditions of culture.

The fungus was grown on a modified Czapek-Dox medium, as used for *P. equinum*.

Activity appeared between the 8th and 15th days and disappeared within 24 h urs. Frequent examination of the culture fluid and rapid extraction of the active principle when produced were essential for the successful isolation of the antibiotic.

Isolation.

The culture fluid was acidified to pH 2 when a precipitate formed carrying with it all the activity. The precipitate was centrifuged, suspended in water and extracted with chloroform. The active principle was extracted from the chloroform into aqueous solution at pH 7·5, and was then precipitated as a yellow microcrystalline solid by addition of hydrochloric acid. It was recrystallized from aqueous alcohol or benzene.

Chemical properties.

Herquein forms yellowish-brown crystals, m.p. 129° C. (decomp.). Found: C 59·84, H 5·42, OCH_3 5·33, $CH_3(C)$ 10·5 per cent. $C_{19}H_{20}O_8$ requires: C 60·6, H 5·2. The substance is sparingly soluble in water, giving a yellow solution which exhibits a bottle-green fluorescence on addition of alkali.

It is readily soluble in ethanol and chloroform, moderately soluble in ether and carbon tetrachloride, slightly soluble in benzene and insoluble in petroleum ether. It can be precipitated from aqueous solution by means of lead acetate, and recovered by decomposing the lead with sodium sulphate.

Antibacterial properties.

Dr. J. S. Robertson tested the crude culture fluid against a wide variety of organisms, and reported activity against *Sh. shigae*, *Myco. phlei*, *Str. pyogenes*, *V. cholerae*, *Staph. albus*, *Staph. aureus* and *Ps. pyocyanea*.

Tested by the wheel plate method, a 1/500 solution of herquein inhibited the growth of the El Tor vibrio, *V. cholerae*, *C. xerosis* and *Staph. aureus*.

In serial dilution tests it inhibited the growth of *Staph. aureus* and *V. cholerae* at 1:2500, but not that of the El Tor vibrio.

Penicillin-like Antibiotics from Penicillia.

The culture fluids of *P. euglaucum*, *P. meleagrinum*, *P. divaricatum*, *P. roseo-citreum* Biourge (Baarn), *P. roseo-citreum* Biourge (N.C.T.C.) and *P. roseo-citreum* (P. 94) were all reported to inhibit the growth of *Staph. aureus* by Miss M. E. Johns.

Conditions of culture.

P. euglaucum was grown on a modified Czapek-Dox medium, as used for *P. equinum*, together with 5 per cent neutralized corn steep liquor. Activity was maximal in about 10 to 15 days.

P. meleagrinum was grown on the above medium. Activity was maximal in 5 to 10 days.

P. roseo-citreum (N.C.T.C. strain) and *P. roseo-citreum* (P. 94) were grown on the above medium, whilst *P. roseo-citreum* (Baarn) was grown using a similar medium, but without the addition of corn steep liquor. Activity was maximal in 10 days.

Extraction.

In all these cases the culture fluid was acidified to pH 2 with hydrochloric acid, and extracted with one-seventh volume of either ether or ethyl or amyl acetates. All the activity was extracted from the culture fluid at pH 2 and none at pH 7 or pH 10. The organic solvent layer was extracted with phosphate buffer at pH 7 when the activity passed entirely into the aqueous layer.

Chemical properties.

In each case activity was totally destroyed by incubation of either the crude culture medium or the extract for two hours at 37° C. with penicillinase or by incubation in the presence of copper sulphate.

The activity was also destroyed by heating the extract at 100° C. for 15 minutes at pH 2 and at pH 9, and was partially destroyed by heating for 15 minutes at pH 7.

It is concluded that these penicillia produce penicillin-like substances.

SUMMARY.

Antibacterial substances have been extracted from the culture fluids of 13 penicillia.

It has been found that six of these, *P. meleagrinum* Biourge, *P. euglaucum*, *P. divaricatum* and three strains of *P. roseo-citreum* produce penicillin-like antibiotics alone, and that two others, *P. baarnense* v. Beyma and *P. bialowiezense* Zal., produce a penicillin-like antibiotic, together with other antibiotics. *P. baarnense* produces penicillic acid, *P. bialowiezense* and *P. viridicatum* produce mycophenolic acid, and *P. equinum* v. Beyma and *P. novae zeelandiae* v. Beyma produce expansine. *P. herquei* N.C.T.C. 1721 produces an antibiotic named herquein, which inhibits the growth of *Staph. aureus* and some vibrios at high concentrations. *P. rubrum* produces a compound which has antibacterial properties which has been shown to be phoenicin.

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